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AUTOTRANSPLANTATION OF THE ADRENAL OF THE RAT TO THE PORTAL CIRCULATION: EFFECT OF ADMINISTRATION OF ACTH[1]

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ABSTRACT

An extraordinary hypertrophy occurs in the adrenal gland transplanted into the portal circulation of the female rat. This has been shown to be eliminated by castration. In the present studies, in spite of the large dosage of ACTH, the hypertrophy attained in the implanted gland of the castrated female was but half of that which occurred when the ovaries were present.

Ordinarily the hypertrophy does not occur in the male. The administration of ACTH to this sex produced only a slight degree of adrenal enlargement which was attributed to the trophic effect of the ACTH and which was not modified by intraportal implantation nor the presence or absence of the testes.

It is concluded that the extraordinary hypertrophy of the adrenal implanted into the portal venous bed is strongly dependent upon estrogen and cannot be reproduced to the same extent by the administration of ACTH.

IN A series of investigations in which adrenal glands were implanted into the portal venous bed of adrenalectomized, non-castrated and castrated rats, we reported an unusual adrenal hypertrophy which occurred exclusively in the non-castrated female (1, 2). In subsequent studies it was shown that estrogen was essential for, and androgen inhibitory to, the production of this hypertrophy in the transplanted adrenal (3). It appeared, therefore, that the development of the hypertrophy was directly affected by gonadal hormones. Although a marked estrogen dependency was demonstrated, it seemed likely that other hormones could also play a positive role in this adrenal enlargement. If, by the administration of an exogenous hormone, the hypertrophy could be induced to any significant extent in the absence of estrogen, it would provide indirect evidence of the influence of the hormone in question. The present investigation was undertaken to

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study the effect of exogenous ACTH on the adrenal implanted into the portal circulation of the rat.

METHODS

Experiment 1. Female rats of the Long Evans strain which weighed approximately 150 grams were separated into 4 groups as follows: In "Group A", which served as a control, the right adrenal was removed and the left retained *in situ*. In "Group B" the right adrenal was removed and the left re-implanted into the portal circulation. "Group C" was identical to "Group A" but in addition was castrated. "Group D" was identical to "Group B" and in addition was castrated.

Experiment 2. Male rats of the same strain which weighed approximately 250 grams were similarly separated into "Group Am", "Group Bm", "Group Cm", and "Group Dm", each of which received the same treatment as the corresponding group of female rats in Experiment 1.

The animals in all 8 groups were injected with adrenocorticotrophin, 2 U.S.P. units intramuscularly, five times per week until autopsy. At the end of 150 days the rats were weighed and sacrified. The adrenal glands were carefully dissected free of adherent tissues and weighed. If adrenal remnants were found due to incomplete removal of the gland from its original site, or if accessory tissue was discovered, the animals were not included in the series.

RESULTS

Experiment 1. In female non-castrated animals (Table 1), the adrenal implanted into the portal venous bed ("Group B") underwent a marked hypertrophy ($P < .001$) compared to the solitary adrenal left *in situ* ("Group A"). Castrated groups C and D demonstrated the same effect; again the adrenal in the portal circulation underwent a marked hypertrophy when compared to the adrenal *in situ* ($P < .001$). However, it is evident that the stimulation of the transplanted adrenal in "Group D" (increment of 10.9 mg) was markedly less than that of "Group B" (24.8 mg) in which the ovaries were present. Comparison of the control animals

TABLE 1. MEAN CHANGE IN ADRENAL WEIGHT IN FEMALE RATS AFTER 150 DAYS OF ACTH ADMINISTRATION*

	Type of experiment	Number of rats	Initial wt. (right adrenal)	Final wt. (left adrenal)	Increment	S.D. of increment
Group A	Left adrenal *in situ*	11	15.0 (24.5)†	25.9 (60.3)	10.9	2.1
Group B	Left adrenal in portal circulation	11	16.5 (29.0)	41.3 (102.6)	24.8	3.8
Group C	Left adrenal *in situ*, castrated	10	11.7 (20.0)	14.5 (41.1)	2.8	1.7
Group D	Left adrenal in portal circulation, castrated	18	13.1 (22.2)	24.0 (64.0)	10.9	1.3

* The weight of the adrenal is expressed as the mean weight in mg. per 100 gm. body weight.

† All numbers in parentheses are the mean actual weights of the adrenals in mg.

TABLE 2. MEAN CHANGE IN ADRENAL WEIGHT IN MALE RATS AFTER 150 DAYS OF ACTH ADMINISTRATION*

	Type of experiment	Number of rats	Initial wt. (right adrenal)	Final wt. (left adrenal)	Increment	S.D. of increment
Group Am	Left adrenal *in situ*	6	6.4 (14.9)†	14.6 (40.3)	8.2	4.9
Group Bm	Left adrenal in portal circulation	6	6.1 (15.7)	11.7 (39.5)	5.6	2.7
Group Cm	Left adrenal *in situ*, castrated	6	7.2 (18.9)	14.2 (58.5)	7.0	1.7
Group Dm	Left adrenal in portal circulation, castrated	11	6.6 (19.5)	15.2 (53.6)	8.6	1.8

* The weight of the adrenal is expressed as the mean weight in mg. per 100 gm. body weight

† All numbers in parentheses are the mean actual weights of the adrenal in mg.

also demonstrated that in the castrated "Group C" the increment of 2.8 mg was markedly less than in the non-castrated "Group A" (10.9 mg) in spite of the administration of ACTH.

Experiment 2. A slight degree of adrenal hypertrophy occurred in all of the males (Table 2). However, the extent of the hypertrophy was statistically of the same magnitude in all groups. The body weights (not tabulated) of the castrated rats were considerably greater than the non-castrated, which probably accounts for the increased "actual" adrenal weights of these animals.

Histological examination of the adrenal glands from all of the rats showed a cellular structure similar to that described in our previous communications.

DISCUSSION

Previous studies showed that the marked hypertrophy which occurred in the adrenal gland implanted into the portal venous bed of the rat was dependent upon estrogen (1, 2, 3). This dependency was demonstrated by the following: a) the hypertrophy occurred only in the female and was eliminated by castration; b) it did not occur in the male sex, and c) it could be induced in the male by the administration of estrogen.

In the present studies, the injection of ACTH in considerable dosage did produce hypertrophy in the implanted gland in the castrated female. However, the extent of the enlargement was only half of that which occurred in the non-castrated female whether ACTH had been administered or not. Moreover in the male rats given ACTH, the implanted adrenal was not hypertrophied to an extent any greater than that of the solitary gland *in situ*.

In accordance with the feed-back mechanism for ACTH production, an

increased secretion of this pituitary trophin would occur if the level of steroids was lowered in the peripheral circulation. Indeed, we demonstrated that the level of steroids in the peripheral blood of non-castrated females with an intraportal adrenal was lower than that in control animals (4). It would be most tempting to assume that "over-production of ACTH" produced the marked hypertrophy of the implanted gland. An indirect test of this hypothesis was performed in an earlier investigation in which cortisone was administered to inhibit an excess production of ACTH. But in spite of the cortisone, the hypertrophy occurred as long as the ovaries were present. Moreover, the excess of ACTH administered in the present studies did not produce a marked hypertrophy of the implanted gland in the male, and only enlarged the gland in the castrated female to half the extent of that which occurred in the non-castrated female. It is concluded, therefore, that "overproduction of ACTH" is neither the sole, nor even the dominant mechanism responsible for the development of the adrenal hypertrophy in the implanted gland. The important influence of estrogen, whether endogenous or exogenous, is yet to be explained.

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THE SPECIFICITY OF THE INTERACTION OF INSULIN-I^{131} WITH TISSUE

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ABSTRACT

Insulin-I^{131} possessing full hypoglycemic activity and insulin-I^{131} totally inactivated either by treatment with dilute NaOH at 37° or by heavy iodine substitution (5.6 I atoms/molecule) were intravenously injected into male albino rats. The following results were demonstrated 5 minutes later. 1) Inactivation caused a marked reduction in the concentration of the labeled protein by the liver and, in marked contrast to the active hormone, permitted large quantities to be removed by perfusion. 2) The proportion of inactivated insulin-I^{131} bound to intracellular structures was reduced by alkali inactivation and increased by heavy iodine substitution; specific alterations were also observed in the pattern of distribution among the intracellular structures (nuclei, mitochondria and microsomes). 3) Degradation by liver and kidney was different for both inactivated insulin preparations. These results support the thesis that the metabolic fate of hormonally intact insulin-I^{131} in the liver is specific. The data are discussed in relation to the validity of the use of insulin-I^{131} as a biological tracer and the relationship between molecular structure and tissue interactions.

INSULIN labeled with I^{131} has been used to elucidate the fate and distribution of native insulin (1, 2, 3). The interpretation of such data requires the assumption that the labeled hormone enters into physiologic processes in a manner based on its unique structural and functional relation to the native protein. Two lines of evidence provide an insight as to the correctness of this assumption. Firstly, the characteristic nature of insulin-I^{131} distribution data has been indicated by control studies with albumin-I^{131}, thyroxine-I^{131} and sodium iodide-I^{131}. However, this is not especially convincing because of the marked physico-chemical dissimilarities, particularly molecular size. The inappropriateness of these controls was confirmed by the fact that distribution values similar to those for insulin-I^{131} could be obtained for ribonuclease-I^{131}, a protein of approximately the same molecular weight (4). The second line of evidence is potentially more powerful since it is concerned with specific protein interactions, namely enzymic degradation by "insulinase" (5) and binding by specific antibodies (6). However, the finding that such insulin-I^{131} preparations contain a number of different iodinated molecular species, differing in the amounts, kinds and intramolecular locations of the iodotyrosine residues, weakens the

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value of this criterion (7). Hence, it is unlikely that a conclusive decision is possible concerning the original assumption and it will probably have to be re-evaluated for each specific parameter of insulin physiology.

The present report is concerned with the validation of previous distribution studies (1, 8) by comparisons with controls of more suitable molecular properties. Various aspects of these studies were repeated using insulin-I^{131} inactivated either by treatment with dilute alkali (9) or by excessive iodine substitution (10).

MATERIALS AND METHODS

Insulin-I^{131}

Insulin-I^{131} was obtained from Abbott Laboratories; such preparations were subjected to *in vivo* bioassay by the Lilly Research Laboratories, by the mouse convulsion test as well as the blood sugar depression in rabbits using a crossover technique, and found to have undiminished hormonal potency (7). Upon receipt it was diluted with 0.001 *M* phosphate buffer, pH 7.4, containing 0.02% phenol and 100 mg.% of sterile human serum albumin, and stored at 0–4° C for no more than 7 days prior to use. Before use it was exhaustively dialyzed against buffer in the absence of phenol and carrier protein, and showed 98–100% of the I^{131} to be precipitated, in the presence of 2% plasma protein, with 5% trichloroacetic acid (TCA).

Inactivated insulin-I^{131} was prepared by two methods. Abbott insulin-I^{131} was treated with dilute NaOH (pH 12.5) at 37° C for 3 hrs., the product possessing no hypoglycemic activity at the end of this time (9) and, after dialysis, being completely precipitable with TCA in the presence of carrier protein. The second method involved reacting crystalline zinc insulin with an aqueous solution of I_2. The iodine had been previously labeled with I^{131} and purified by solvent extraction. The resulting iodoprotein was carried through several cycles of acid solution and isoelectric precipitation so that the final product showed no detectable I^{131} not bound to protein (10). It contained, on the average, 5.6 I atoms/insulin molecule (6000 MW), had no hypoglycemic activity and showed the same TCA precipitability as the other materials.

Experimental procedures

The animals were male albino rats of the Sprague-Dawley line, weighing 200–250 grams and fasted 16–18 hours before use. Under light ether anesthesia the radio-active preparation was injected into one of the tail veins in a volume not greater than 0.5 ml. In the case of active insulin-I^{131} the dose ranged from 2.7×10^{-2} to 7.0×10^{-2} units (1.0–2.6 μg.) and 2.7 to 4.3 μc. In the case of the inactivated preparations the same radiation doses were used but the protein ranged from 6.5 to 11.3 μg. In the parameters of these studies, no alternations were observed that could be attributed to dosage; this finding was consistent with previous reports (4, 8). Five minutes later, under deep anesthesia, the blood was collected in heparin from the abdominal aorta, and the liver and kidneys were removed. After rinsing in cold isotonic KCl the organs were homogenized and aliquots assayed directly for total I^{131} activity using a well-type scintillation counter. Additional aliquots were treated with TCA, the precipitates washed and digested in 30% KOH, and samples of the digests assayed for I^{131} content; the difference between this value and the total I^{131} corresponded to the amount of insulin-I^{131} degraded into fragments not precipitable with 5% TCA. Similar procedures were carried out for the heparinized plasma. This method, that of TCA precipitation, results in analytic samples minimally contaminated with radioactive digestion products (1, 11). On the other hand, TCA precipitation makes no distinction between intact insulin and that altered by

radiation effects coincident to the preparation and nature of these materials (12, 14). Two relevant factors should be pointed out, however. Firstly, due to the early sampling times (5 minutes), detection of such physical differences would not have been possible by electrophoretic or hydrodynamic flow techniques (12, 15). Secondly, the method of preparation, storage and handling of the insulin-I^{131} was such as to render the effects of radiation negligible (13, 14).

A number of studies were carred out relative to the intracellular distribution of radioactivity. The livers were perfused and homogenates prepared with cold 0.25 *M* sucrose and subjected to a differential centrifugation schedule somewhat different from that previously used (8, 10). The resultant specimens corresponded to a washed nuclear (N_w), a washed mitochondrial (M_w), a microsomal (M_c) and a residual fraction; this last contained all of the TCA-soluble I^{131} found in the initial homogenate.

In order to assess the role of passive adsorption of the labeled proteins to particulate elements, several additional groups of determinations were carried out in which the radioactive preparations were added to liver homogenates prior to centrifugation. Such determinations are referred to in the text and tables as the *in vitro* series in contrast to those determinations in which the labeled protein had been used intravenously (*in vivo* series).

All radioassays were carried out under fixed conditions of geometry and enough counts collected to keep the error less than $\pm$ 2%. The resultant counting values were corrected for decay when necessary and for differences in body weight by multiplying by a correction factor (body weight in grams/200).

RESULTS

The concentration of insulin-I^{131} by liver and kidneys. Figure 1 shows the

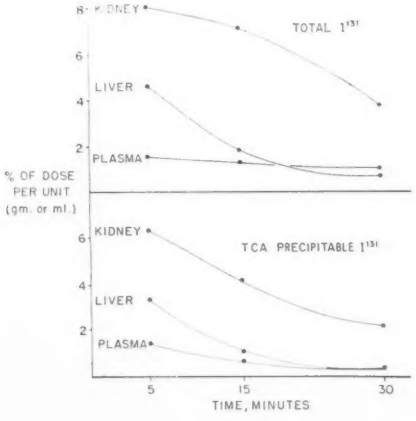

FIG. 1. The distribution of radioactivity in the rat following insulin-I^{131} injection.

values for the total and TCA-precipitable I^{131} of liver, kidney and plasma during the 30 minutes immediately following the intravenous injection of insulin-I^{131} possessing full hormonal activity. These data agree with that reported by others (1) and indicate a marked concentration of the labeled hormone by liver and kidney. In order to evaluate the specificity of this process, comparisons for the active and inactivated (NaOH and I_2) prepa-

TABLE 1. THE DISTRIBUTION OF I^{131} IN THE RAT FIVE MINUTES AFTER THE INTRAVENOUS INJECTION OF INSULIN-I^{131}

	n	% of the dose/Unit of tissue						Organ:Plasma ratio of total I-131	
		Liver		Kidney		Plasma			
		Total	TCA ppt.	Total	TCA ppt.	Total	TCA ppt.		
Active Insulin-I^{131}	17	4.54 ±0.54*	3.09 ±0.45	8.06 ±1.02	6.19 ±0.97	1.51 ±0.25	1.31 ±0.25	3.01	5.34
Inactive Insulin-I^{131} NaOH	17	1.51 ±0.20	1.14 ±0.27	7.11 ±0.92	4.03 ±0.48	2.42 ±0.25	1.81 ±0.32	0.62	2.94
I_2	8	2.65 ±0.15	2.40 ±0.18	6.00 ±0.45	5.63 ±0.37	2.49 ±0.18	2.49 ±0.18	1.06	2.41

* Mean ± standard deviation

rations are presented in Table 1, the time after injection being 5 minutes. Marked reductions in concentration are particularly apparent, ranging from 22–62% for liver and 12–33% for kidney. Since many factors may contribute to these reductions, the specificity of the concentrative activities of the 2 organs may be more exactly expressed by relating their values to the availability of the labeled material; the organ-plasma ratios provide such an expression. In the case of liver, NaOH-inactivation reduced the organ:plasma ratio to $\frac{1}{5}$ and with excessive I_2 substitution to $\frac{1}{3}$ of control values; a qualitatively similar picture was obtained for kidney.

Perfusion studies with liver. Perfusion has provided an experimental method for revealing the firm *in vivo* union of insulin-I^{131} with tissue (1, 8). In this connection total-I^{131} values are entirely inappropriate since they also represent the products of labeled hormone degradation. In order to dispense with this limitation the data for the perfusion experiments reported in Table 2 are only for TCA-precipitable I^{131}, hence more exactly represent the labeled protein molecule. It is quite apparent that perfusion removed none of the labeled protein from the liver when the functionally intact hormone was injected whereas almost 40% was lost when the preparations had been inactivated with NaOH or excessive I_2 substitution. This should be compared with similar experiments using I^{131} labeled human serum albumin; in this case the loss was greater than 60% (8).

TABLE 2. THE EFFECT OF INACTIVATION ON THE BINDING OF INSULIN-I^{131} TO RAT LIVER

	Insulin-I^{131} concentration (% of dose/gm. in TCA precipitable fraction)				% removed by perfusion
	n	Unperfused	n	Perfused	
Active Insulin-I^{131}	17	3.09 ±0.45*	50	3.15 ±0.38	0
Inactive Insulin-I^{131}					
NaOH	36	1.14 ±0.27	42	0.68 ±0.19	36
I_2	6	2.40 ±0.18	12	1.52 ±0.19	37

* Mean ± standard deviation.

These results are not completely unequivocal, however, if one takes into consideration the insulin-I^{131} in the plasma removed by organ perfusion. Using the data of Everett, Simmons and Lasher for the size of rat organ blood and plasma compartments (16), it can be calculated that, although 36% of the TCA-precipitable I^{131} in the liver was removed by perfusion in the NaOH series, 30% could have been in the trapped plasma of that organ. This possibility greatly weakens the value of these observations relative to the interpretation that intracellular iodo-protein had been removed. However, similar calculations for the I_2-inactivated series resulted in a value of 19% for the occluded plasma in contrast to a 37% loss in perfusion; in this

TABLE 3. THE EFFECT OF INACTIVATION ON THE BINDING OF INSULIN-I^{131} TO CYTOSTRUCTURAL ELEMENTS OF RAT LIVER

	n	% dose/gm.	Binding to cytostructures			
			% in tissue	Difference from active		
				%	"t"	P
In vivo						
Active Insulin-I^{131}	32	3.16 ± 0.42*	73.2 ± 4.5	—	—	—
Inactive Insulin-I^{131}						
NaOH	20	0.69 ± 0.21	66.2 ± 5.0	−7.0 ± 4.7	4.99	<0.001
I_2	12	1.52 ± 0.19	77.1 ± 4.2	+3.9 ± 4.4	2.60	<0.02
In vitro						
Active Insulin-I^{131}	6	—	25.6 ± 1.4	—	—	—

* Mean ± standard deviation.

case the inference of intracellular removal remains essentially unshaken. Again, similar calculations for the case of intact insulin-I^{131} indicate the TCA-precipitable I^{131} of the afferent plasma to be largely, if not completely, removed.

Intracellular distribution studies. The specificity of insulin-I^{131} binding to liver, indicated by the concentration and perfusion data reported here and elsewhere (1, 3, 8), was investigated by determining the amount of active or inactivated insulin-I^{131} bound to cytostructural elements. Data relative to this are presented in Table 3. In addition to the *in vitro* studies with the active hormone, similar studies were carried out for both inactivated preparations; since the results were found to be the same in all cases, only the values for the active preparation were presented in the table.

It is quite apparent that *in vivo* injection resulted in approximately 3 times as much TCA-precipitable I^{131} being bound to cytostructures as could be attributed to passive adsorption following the addition of the labeled proteins to a homogenate. The firmness of this binding has been examined by both perfusion and washing experiments and it has been shown that no radioactivity could be removed for the *in vivo* case whereas almost all could be removed for the *in vitro* case (10). The specificity of this

in vivo binding was indicated by the statistically significant reduction in the case of one type of molecular alteration (NaOH inactivation) as compared to a statistically significant increase resulting from the other type of molecular change (excessive I_2 substitution). This aspect of specificity was further emphasized by the differences in intracellular distribution found to be associated with the method of hormone inactivation (Table 4). Again, the pattern for *in vitro* adsorption was markedly different from the *in vivo* case, thus vitiating the claim for passive adsorption as an important mech-

TABLE 4. THE EFFECT OF INACTIVATION ON THE INTRACELLULAR DISTRIBUTION OF INSULIN-I^{131} IN RAT LIVER

	n	% dose/gm.	Binding to cytostructures			
			% in tissue	Difference from active		
				%	"t"	P
In vivo						
Active Insulin-I^{131}	32	3.16±0.42*				
Nw			7.0±1.6	—	—	—
Mw			17.3±3.3	—	—	—
Mc			48.9±4.5	—	—	—
Inactive Insulin-I^{131}						
NaOH	20	0.69±0.21				
Nw			6.7±0.9	− 0.3±1.6	0.675	>0.5
Mw			20.9±2.7	+ 3.6±3.1	3.980	<0.001
Mc			38.6±3.5	−10.3±4.2	8.350	≪0.001
I_2	12	1.52±0.19				
Nw			6.3±1.7	− 0.7±1.7	1.240	>0.2
Mw			20.2±3.2	+ 2.9±3.3	2.600	<0.02
Mc			50.6±5.4	+ 1.4±4.8	1.040	>0.3
In vitro						
Active Insulin-I^{131}	6	—				
Nw			7.4±0.7	—	—	—
Mw			2.7±0.2	—	—	—
Mc			15.4±1.3	—	—	—

* Mean ± standard deviation.
Nw = washed nuclear fraction, Mw = washed mitochondrial fraction, Mc = microsomal fraction.

anism (17). Also, such distribution values were different for the inactivated preparations. In the case of the NaOH method the increases in the proportion of mitochondrial-bound I^{131} and the decrease for microsomal-bound I^{131} were highly significant whereas for the I_2-inactivated material only the increase in mitochondrial-I^{131} was statistically significant.

Degradation studies. The ultimate fate of insulin-I^{131} was considered from the standpoint of its molecular degradation by the two organs most quantitatively important in this regard, liver and kidney (18). This was done by determining the TCA-precipitable fraction of the I^{131} present in unperfused liver and kidney; such values are presented in Table 5. Qualitatively, the same conclusions apply to both organs. Inactivation resulted in differences in the degradation of the labeled protein and the nature of the difference, relative to the values for the active hormone, was determined by the

method. Five minutes after injection proportionately less of the NaOH-inactivated insulin-I^{131} in the two organs remained undegraded whereas the opposite was true for I_2-inactivation.

DISCUSSION

It had been concluded in earlier studies that the fate and distribution of insulin-I^{131} in the rat were specific for that protein and indicative of the fate

TABLE 5. THE EFFECT OF INACTIVATION ON THE DEGRADATION OF INSULIN-I^{131} IN RAT LIVER AND KIDNEY

	n	Per cent of total I^{131} in TCA precipitable fraction	
		Liver	Kidney
Active Insulin-I^{131}	17	68 ± 4.0*	77 ± 5.5
Inactive Insulin-I^{131}			
NaOH	17	60 ± 7.5	46 ± 6.8
I_2	8	91 ± 4.2	94 ± 2.8

* Mean ± standard deviation.

and distribution of the intravenously administered unlabeled hormone. Numerous observations have been presented supporting the validity of this conclusion but it was felt that they did not offer the most convincing comparisons since the test substances used differed so fundamentally from the protein of interest (1, 8, 19). The present study was designed to correct this complaint by executing similar comparison studies using insulin-I^{131} altered sufficiently to result in complete loss of hormonal potency. One method of alteration, using dilute NaOH, results in a small loss of nitrogen, presumably from amide groups and not of significance in affecting potency (20), and the conversion of some of the cystine residues to lanthionine (21); it is probable that this latter change is responsible for the loss in potency (22). The other method, also resulting in a complete loss in potency, operates by substitution on the tyrosine residues exclusively (7, 20). Under the conditions employed here, the heavily iodinated preparations had over 99% of the molecules substituted several times with most iodotyrosine residues present as diiodotyrosine (10).

The results presented in this paper may be organized as follows. When sufficient damage had been inflicted on the insulin-I^{131} molecule to result in a complete loss in hormonal potency, its fate in the rat was distinctly altered. Concentration of total I^{131} by liver and kidney was reduced as well as the fraction considered to represent the intact molecule (TCA-precipitable). Furthermore, binding to liver was less firm and the amount and distribution of that bound to intracellular structures were changed. Lastly, the degradation by tissue enzyme systems was altered. Hence, it may be considered that these observations validate the earlier conclusion that the

fate and distribution of insulin-I^{131} in the rat are characteristic and specific for the functionally intact molecule.

Before leaving this aspect entirely, however, the issue of the validity of use of insulin-I^{131}, possessing full potency, as a tracer of the native protein needs discussion. This question is raised by the very results supporting the conclusion of specificity since it is concerned with the quantitative aspect of molecular alteration in causing changes in the biologic parameters. This question has been engaged elsewhere and the concensus is that, although insulin-I^{131} is an artificial iodoprotein, no evidence has been brought forth inconsistent with its role as tracer of native insulin; this has been shown in relation to metabolic, immunologic and bioassay criteria for preparations possessing undiminished hormonal potency (1, 4, 6, 7). The present concern may be resolved into an experimentally feasible problem: to determine the relationship between the extent of I substitution and hormonal potency. This has been investigated with the result that it was found necessary for substitution to exceed, on the average, 2 I atoms/molecule before potency loss could be detected (7). Since this ranges from 4–6 times the level of substitution commonly used, it does not seem reasonable to believe the various conclusions obtained in this paper to be valid only for iodoinsulin and not for native insulin.

The interesting possibility raised by these data is that an experimentally demonstrable relationship may exist between the binding to cytologic particulates, tissue degradation and molecular structure. It is apparent from Tables 3 and 5 that NaOH inactivation resulted in proportionately less binding to liver cytostructures (66.2% *vs* 73.2%) and proportionately greater degradation (60% *vs* 68% TCA precipitability) as compared to the active material whereas I_2 inactivation resulted in an increased intracellular binding (77.1% *vs* 73.2%) and a diminished degradation (91% *vs* 68% TCA precipitability). Basic to these relationships, however, is the question of the role of binding to cytostructures in the degradation processes; namely, does cytostructural binding inhibit or facilitate degradation. Investigation of the particular question has been conducted with regard to the mitochondrial and microsomal populations of liver cells, and it was shown that insulin-I^{131}, bound to mitochondria or microsomes, was very much less susceptible to degradation than the fraction in solution (10). In this regard it should be noted that 50% of the TCA-precipitable activity is bound to the microsomal fraction (Table 4), hence changes in binding to this population should have a large determinant effect.

These investigations have also brought into focus another interesting consideration. The proportion of intracellular insulin-I^{131} bound to cytostructural elements varied over a fairly small range (66.2%–77.1%) for the 3 types of preparations reported here whereas the per cent of the total dose per gram of liver bound to such structures showed a much greater range

(0.46%–2.31%). This disparity in range (17% *vs* 500%) cannot be understood in terms of the experimental maneuvers employed here. However, it should be remembered that no consideration has been given to factors other than molecular structure and binding to particulates. It should be evident on *a priori* grounds that some measure of selective membrane transport and permeability must also be operative; this might be at the level of transcapillary exchange as well as parenchymal cell membranes. The data presented in Table 2, as discussed in relevant section of "Results," support this view, both with regard to the removal of the various forms of insulin-I^{131} from the plasma as well as the possible intracellular losses coincident with perfusion. Once inside the cell, binding to cytostructures occurs, the proportions being related to the molecular properties of the iodoprotein; in this regard, the amount getting inside the cell must be under some similar specification. Since the effectiveness and duration of action of therapeutic insulin is, in part at least, determined by its rate of destruction, the investigation of factors concerned with liver and kidney cell wall permeability may have consequences of practical clinical importance.

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HISTOCYTOLOGIC CHANGES IN GRAFTS OF RAT PITUITARY ON THE KIDNEY AND UPON RE-TRANSPLANTATION UNDER THE DIENCEPHALON[1]

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ABSTRACT

Histocytologic comparisons were made, by means of the periodic-acid Schiff (PAS) and the aldehyde fuchsin (AF) techniques, between pars distalis autografts on the kidney and similar grafts re-transplanted close to the median eminence. Time intervals from transplantation to autopsy ranged from 1 day to 6 months.

Transplants to the kidney are severely damaged by the transfer, the functional parenchyma of the established grafts apparently being derived from a thin shell of healthy tissue surrounding the massive central infarct. Reorganization of the graft is essentially complete after 1 week. Normal cytologic differentiation is promptly lost. Large gonadotrophs (PAS+, AF−) are missing, but smaller ones may be found in significant numbers for 2 to 3 weeks; after that they are rarely recognized. Thyrotrophs (PAS+, AF+) are prominent during the first week, but older grafts usually contain in this category only a few minute, angular basophiles and occasional vacuolated cells thought to be "thyroidectomy cells." Small chromophobes are abundant.

Grafts re-transplanted under the median eminence suffer apparently greater loss than at the first operation. But in spite of this, such grafts obtained at autopsy several months later have recovered much of their normal cytologic characteristics, especially in animals with renewed gonadotropic functions. The return of large gonadotrophs is the outstanding feature, with "castration cells" dominating in animals whose ovaries remain inactive. Large thyrotrophs are also common. Techniques were not adequate for extensive evaluation of changes in the acidophile population.

IN A functional study of hypophysial autografts placed under the kidney capsule, Everett (1) observed that they lose much of their cellular differentiation as early as 8 days following transplantation. We later noted, however, in the course of preliminary efforts at re-transplanting such grafts from the kidney to the region of the median eminence (2), that a pro-

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nounced recovery of the cellular differentiation took place. In both investigations the emphasis was on physiology; cytologic study of the grafts was limited to material stained by the routine hematoxylin Mallory tri-acid technique.

More adequate material was made available in the recent amplification of the re-transplantation experiments (3). Using the periodic acid-Schiff and the aldehyde fuchsin techniques we carried out an extensive comparative study of the cytology of pars distalis grafts on the kidney, grafts under the median eminence, and others under the temporal lobe of the brain. The changes while in the kidney site were studied as a function of time to determine how soon the gland loses its characteristic cellular differentiation. This time was then chosen as the shortest interval for the grafts to remain on the kidney before re-transplantation. The principal subject of this communication is the extent to which functional restoration in re-transplanted glands is accompanied by cytologic recovery.

MATERIALS AND METHODS

Sixty-one rats are represented in this investigation, 29 of them from the foregoing study of functional aspects of re-transplantation (3). The reader is referred to that paper for details of the transplantation technique and other experimental procedures. All rats were adult females of the locally inbred Osborne-Mendel strain, $3\frac{1}{2}$ to 7 months old at the beginning of the experiment. All were normally cycling at the time.

The grafts will be considered in three groups. *Group Ky* consists of 33 hypophysial autografts which were left on the kidney for times varying between 24 hours and 94 days before they were removed for examination. Five of these grafts came from animals which served as controls in the study of function (3). *Group MEm* consists of 27 grafts which, after having remained under the kidney capsule for a certain period of time, were re-transplanted under the median eminence. Two of these were removed for examination 24 hours later, one was taken out after 7 days, while the remaining 24 grafts were left in that location for much longer periods. These 24 grafts belonged to the two groups of animals MEm-1 and MEm-2 described in the earlier paper (3). Out of 24 animals which bore these grafts, 20 showed marked restoration of gonadotropic function, and 16 of them had vaginal cycles. In Group MEm-1, the grafts were left on the kidney for 29–33 days, were then re-transplanted under the median eminence and were kept there for the duration of the experiment, 61 to 177 days. The grafts of Group MEm-2 stayed on the kidney for 15 to 21 days after which they were placed under the median eminence for 66 to 173 days. In *Group TL*, 11 grafts were left under the kidney capsule for 17 to 26 days and were then re-transplanted under the temporal lobe of the brain. One graft was removed for study after 24 hours, while the other 10 were left in place from 40 to 78 days (3).

At autopsy, all grafts were observed *in situ* with a dissecting microscope. In the case of grafts under the median eminence, their location on the under surface of the brain was recorded by a sketch; they were then removed with a small block of the overlying hypothalamus. The grafts on the kidney were peeled from the surface of that organ and were completely freed of adhering pieces of the kidney capsule. The grafts under the temporal lobe were detached from the overlying brain before fixation. All grafts were fixed for a minimum of 5 hours in Elftman's chrome alum fixative (4) and were then washed overnight. Serial paraffin sections were cut at 5μ. Two staining techniques were used, the periodic-acid Schiff (PAS) hematoxylin method described by Purves and Gries-

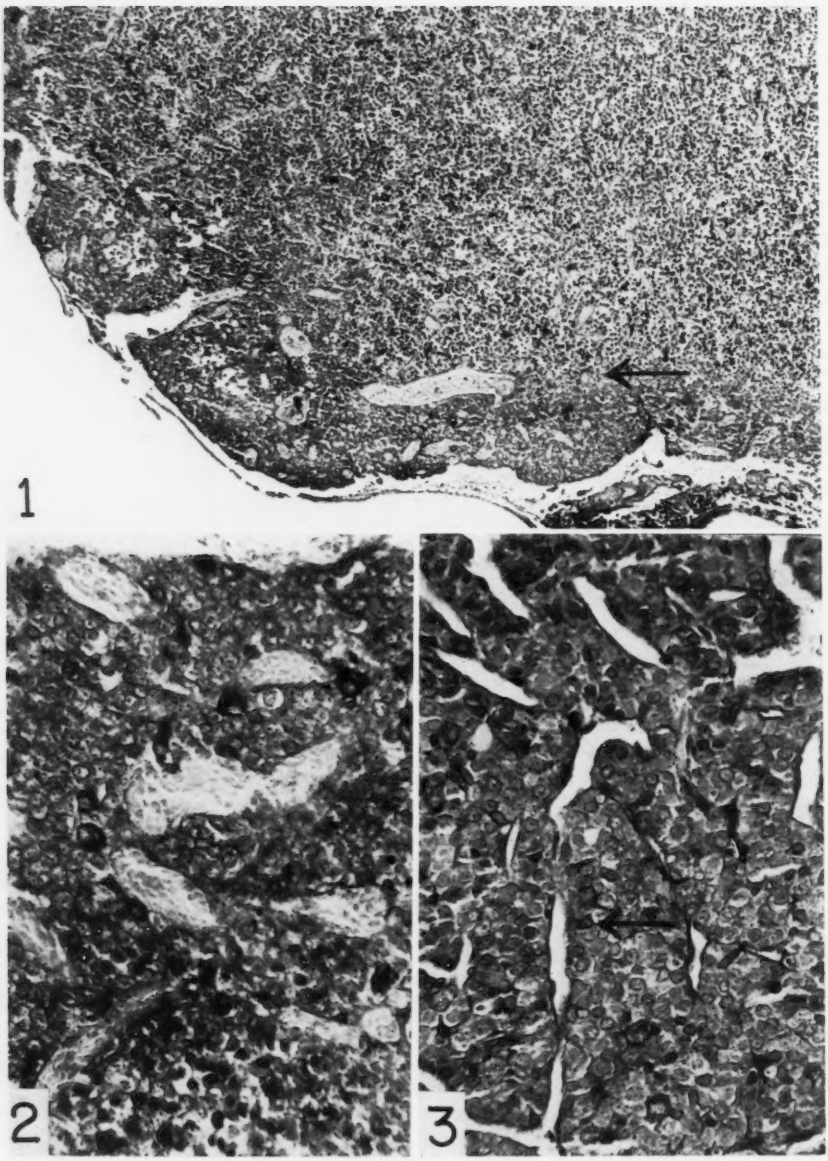
bach (5) and Halmi's aldehyde fuchsin (AF) trichrome stain (6). Since the PAS technique stains all the "basophiles" and AF stains only thyrotrophs, it is necessary to compare adjacent sections stained by the two techniques in order to determine which of the PAS cells are gonadotrophs and which are thyrotrophs. To permit that comparison, successive strips of 5 sections each were mounted alternately on two separate slides until no more tissue remained. At the beginning of the first row of each slide, 2 or 3 sections of normal rat pituitary were mounted to give a reference for the quality of staining. One of each pair of slides was stained with the PAS and the other with the AF technique.

The terminology of Purves and Griesbach (5) is used in the descriptions which follow.

RESULTS

Grafts to the kidney capsule. Twenty-four hours after transplantation of the pars distalis to the kidney capsule the grafts appeared very turgid on direct observation. Histologic examination disclosed a profound loss of parenchyma; although the grafts were large, most of the interior was infarcted and healthy glandular tissue remained in only a thin shell (Fig. 1). Numerous gonadotrophs and thyrotrophs were found here, but the former were already reduced in size. Sinusoids throughout the graft were greatly distended and engorged with erythrocytes (Fig. 2). In the course of the following 3 days, the volume of the infarcted zone was considerably reduced. Although the whole graft was then decidedly smaller, the shell of healthy glandular tissue was thicker, owing at least in part to the reduced circumference. In addition, during the first week, abundant mitoses were present in slender tongues of parenchyma extending into the central necrotic area. Mitotic activity decreased by the end of the week, however, and could not be detected thereafter. From the eighth day onward, there was little change in the histologic aspect of the grafts, even after several months. All such grafts were composed mostly of glandular tissue, and the formerly large necrotic core had become reduced to a rather thin scar. The sinusoids were less engorged with blood than they were during the first few days (Fig. 3).

During the first week, "basophiles" remained rather numerous, but only the thyrotrophs remained large. Surprisingly, 16 or 17 days after transplantation, small gonadotrophs were still fairly abundant (Fig. 4), while the thyrotrophs were scarce. From that time on (except for one 29-day old graft retaining frequent thyrotrophs), both cell types appeared only occasionally. While any gonadotrophs present were always very small, some of the thyrotrophs were of fairly normal size. In these older grafts occasional vacuolated cells were seen, which were interpreted as thyroidectomy cells by virtue of their staining characteristics and morphology (Fig. 5). Usually, they were observed with greater ease in sections stained with AF than in those stained with the PAS technique. In most instances, these cells had an exceedingly thin sheet of cytoplasm about a single vacuole which nearly filled the cell. After the first month there was little change, and 94-day old grafts were practically identical to those seen at 21 to 33 days (compare Figs. 5 and 6).

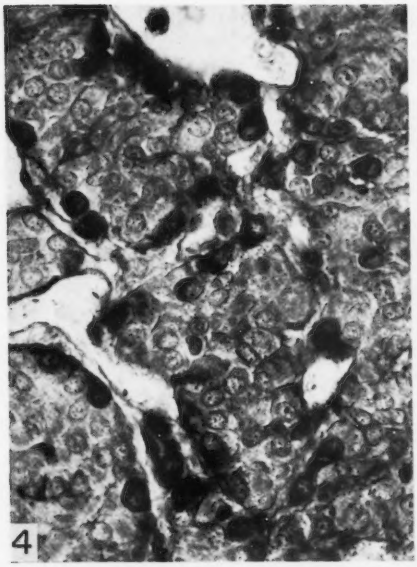


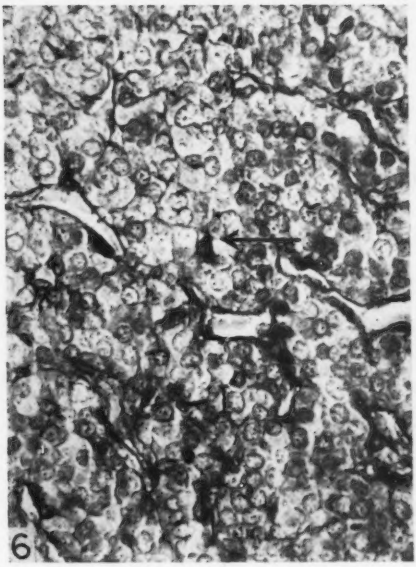
FIGS. 1 to 3. Pars distalis autografts on the kidney, showing early changes.

FIG. 1. One day after transplantation. A massive infarct infiltrated with leucocytes occupies most of the interior, leaving only a thin shell of healthy parenchyma (below arrow). Sinusoids engorged with blood. PAS. 100×.

FIG. 2. Higher magnification of the peripheral zone of the graft represented in Figure 1. Free surface above. Numerous PAS+ cells line the distended sinusoids. 400×.

FIG. 3. Eight days after transplantation. Most of the graft is now constituted by well-organized, healthy, glandular tissue. Note the change in the sinusoids. The arrow points to a small PAS+ cell. 400×.





FIGS. 4 to 7. Well-established pars distalis grafts on the kidney (Figs. 4, 5, 6) and under the temporal lobe of the brain (Fig. 7). All magnifications: 540×.

FIG. 4. At 16 days after transplantation. Small gonadotrophs are still frequent; in the region photographed they are especially numerous. PAS.

FIG. 5. At 30 days after transplantation. Arrows point to a minute, angular cell stained with aldehyde fuchsin (thyrotroph?) and to the large vacuole of a presumptive thyroidectomy cell. Aldehyde fuchsin.

FIG. 6. At 94 days after transplantation. Little different than at 30 days. Note the small, angular PAS+ cell at the arrow.

FIG. 7. At 40 days after re-transplantation, control group TL. Essentially like the grafts in Figures 5 and 6. PAS.

Although the orange G in the AF-trichrome technique stains the acidophiles of the rat hypophysis, our preparations were not consistently satisfactory in this respect. Nevertheless, some changes in this cell type were obvious. Within 3 to 4 days after transplantation, their cytoplasmic volume was greatly diminished, for example. Chromophobes also tended to be small.

Grafts under the median eminence. In all cases of this group the grafts were placed under the median eminence and were adherent to it. Microscopic examination readily disclosed many blood vessels connecting the median eminence and the remainder of the pituitary stalk with the graft (Fig. 8).

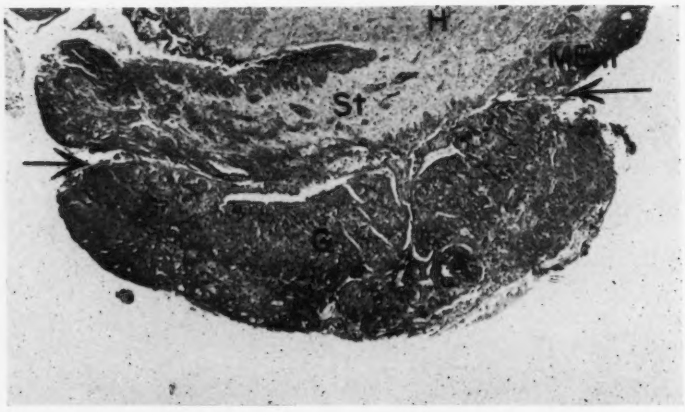
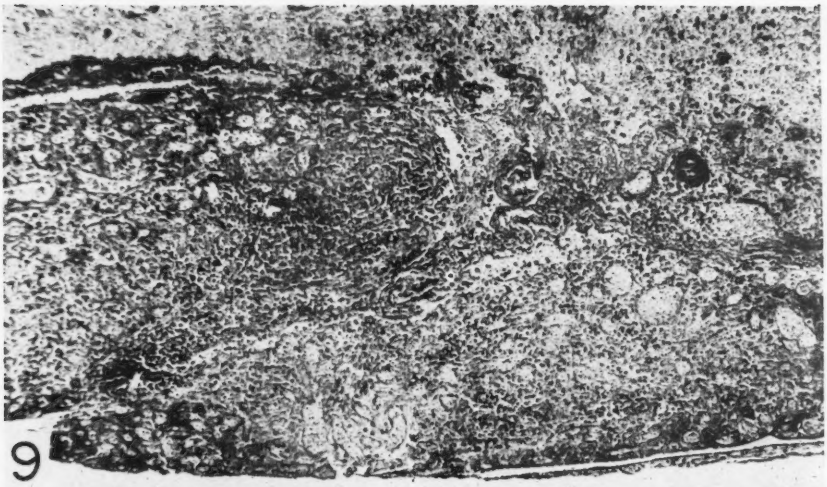
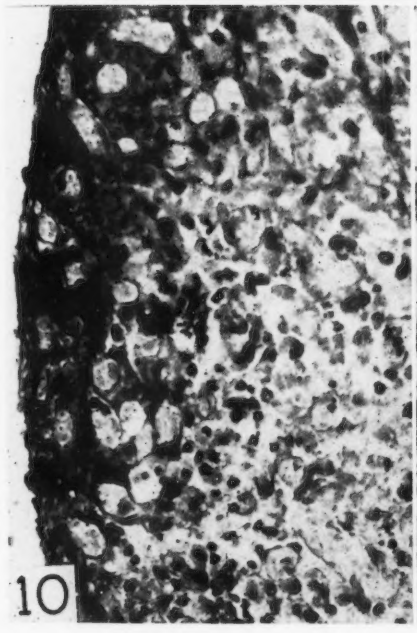
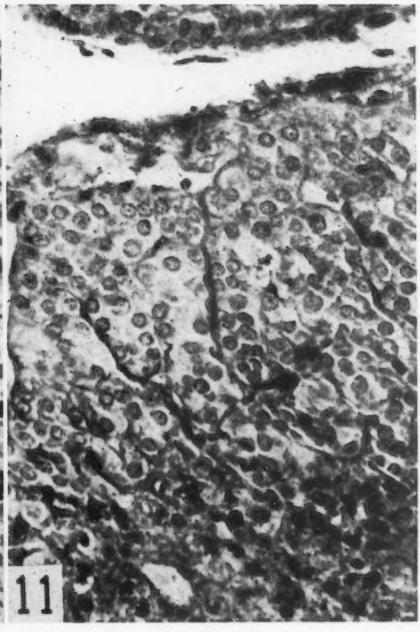


FIG. 8. Typical placement of a pars distalis graft re-transplanted under the median eminence (MEm). The graft (G) had established close contact with the median eminence and the infundibular stem (St) along the line indicated by the arrows. A portion of the hypothalamus (H) is visible above. During most of the 3 months since the operation this animal cycled, but remained sterile. The graft is composed of healthy glandular tissue with the exception of the slender scar (occupied by the many mast cells which appear as small, black spots). Note the epithelial cyst containing colloid darkly stained by the PAS procedure. Parasagittal section. 50×.

The 2 grafts that were studied 24 hours following re-transplantation under the median eminence appeared very turgid, similar to the 24-hour grafts on the kidney. As judged from these examples, this second transplantation was even more harmful to the gland than was the first one (Fig. 9), for histologically the graft was seen to be almost completely infarcted and the shell of healthy glandular tissue was exceedingly thin. Aside from the few small cells found there, most of the parenchymal cells had pycnotic nuclei and were obviously degenerate (Fig. 10). Even at this early date, the union between graft and median eminence was intimate and seemingly completed (Fig. 9). The sinusoids in the graft were wide open and packed





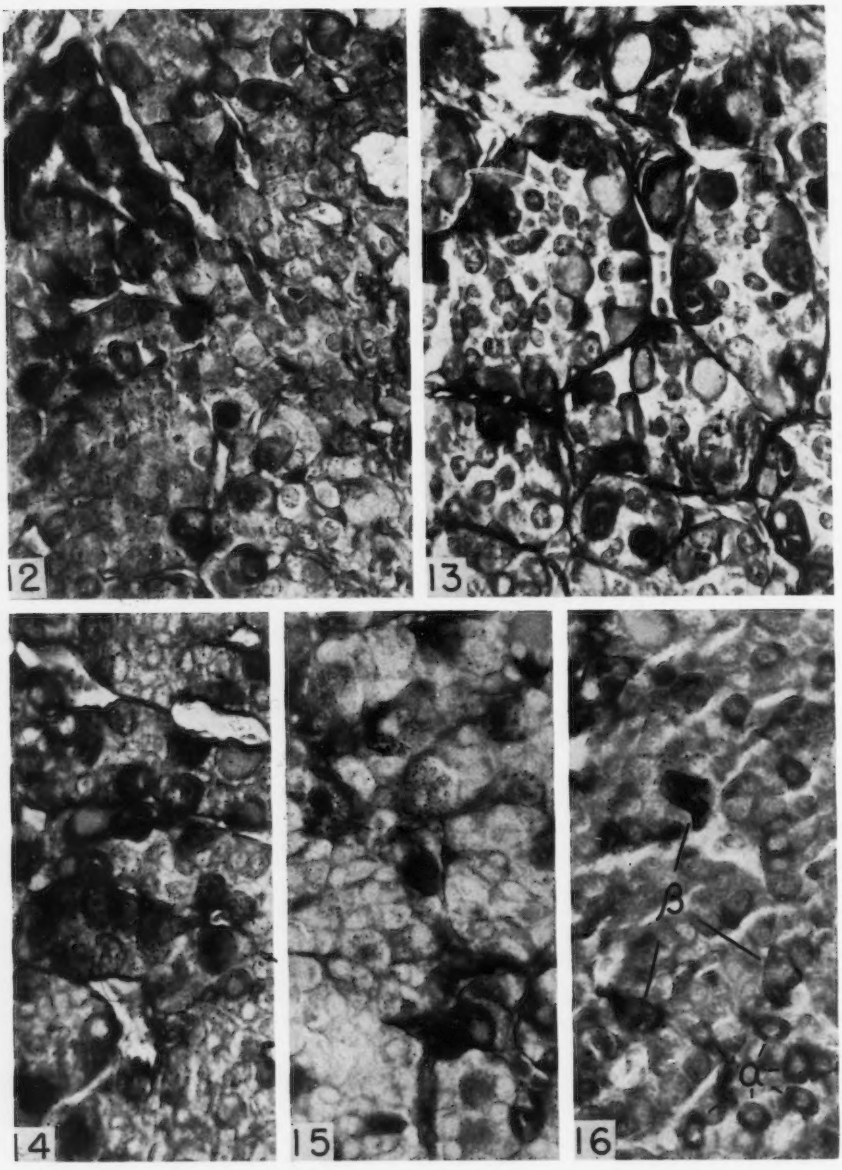


FIGS. 9 to 11. Pars distalis autografts re-transplanted under the median eminence, showing early changes. Compare with Figures 1 to 3.

FIG. 9. One day after re-transplantation. Central infarction has again occurred, this time leaving only a barely perceptible shell of healthy parenchyma. Union with the diencephalon (above) is already intimate. Note engorged sinusoids. PAS. 100×.

FIG. 10. Higher magnification of a portion of the under surface of the graft illustrated in Figure 9, now turned 90°. Note the limited amount of sound tissue along the free surface. PAS. 400×.

FIG. 11. Seven days after re-transplantation. At the very top of the picture are a few cells of pars tuberalis underlying the median eminance. The amount of pars distalis parenchyma has considerably increased, especially on this side of the graft apposed to the infundibular stem. There are numerous large, pale-staining cells in this region; in adjacent sections mitotic figures were noted in several of these. PAS. 400×.



FIGS. 12 to 16. Examples of various cell types in well established pars distalis grafts three to five months after re-transplantation under the median eminence. Contrast with Figures 5, 6, and 7. All magnifications: 565×.

FIG. 12. Abundant large gonadotrophs. From an animal having regular cycles, although not fertile. PAS.

with erythrocytes, while the capillaries of the median eminence appeared to be of normal caliber.

By contrast, a striking improvement was noted in the re-transplanted graft that remained under the median eminence for 7 days (Fig. 11). Although a rather large scar still occupied the interior of the graft, the peripheral parenchyma was well repaired. There were no cells that stained with either the PAS or AF techniques, but numerous large, lightly stained cells with finely granular cytoplasm were present. In a few instances, the characteristic negative image of the Golgi apparatus identified them as "basophiles" in spite of the absence of specific granulation. These enlarged cells, as well as mitotic figures, were found exclusively in regions close to the median eminence.

In all grafts from groups MEm-1 and MEm-2, renewed differentiation of cells was pronounced. Large gonadotrophs (PAS+, AF−) and thyrotrophs (PAS+, AF+) appeared in great numbers (Figs. 12 to 16). There was a strong tendency for cells of a given type to congregate in local clusters. Gonadotrophs were usually far more numerous than the thyrotrophs. Although in some grafts the latter were extremely rare, the same grafts clearly had TSH function, made evident by the thyroidal I^{131} uptake study (3). The re-differentiated cells were usually larger than normal and frequently had an unusually prominent Golgi apparatus. The cytoplasm of various cells stained with different intensity, indicative of a variable amount of glycoprotein in their granules.

In grafts from animals which developed irregular cycles following retransplantation, vacuolated "signet-ring" cells were abundant (Fig. 13), in addition to normal appearing gonadotrophs. Occasional cells of this type were sometimes found even in grafts from animals with regular cycles. In grafts of the one animal from group MEm-1 and the 3 animals from group MEm-2 that never showed cyclic vaginal changes following retransplantation, castration cells were predominant and normal gonadotrophs were only rarely found.

FIG. 13. A population including many large, vacuolated gonadotrophs as well as nonvacuolated ones. From an animal which had prolonged, irregular cycles. PAS.

FIG. 14. Occasional vacuolated cells among numerous normal gonadotrophs. From a fertile animal, cycling, five weeks after delivery. PAS.

FIG. 15. A cluster of thyroidectomy cells in the upper half of the photograph, of both the vacuolated type (extreme upper right) and the type containing intensely stained "T" granules. The latter were never encountered in grafts distant from the median eminence. From an animal which had irregular, prolonged cycles; thyroids of normal weight, but with an I^{131} uptake 40% of normal average. Aldehyde fuchsin.

FIG. 16. Thyrotrophs (β) and acidophiles (α) of normal appearance in an aldehyde fuchsin preparation. From a fertile animal three weeks after delivery; thyroids of normal weight, but with an I^{131} uptake 23% of normal average. Other parts of this graft contained significant numbers of thyroidectomy cells.

In addition to or instead of normal thyrotrophs, thyroidectomy cells were observed in most grafts of this group and occasionally were present in considerable numbers. In some cases they contained extensive hyaline vesicles and in others, large, intensely stained bodies (Fig. 15) corresponding to the "T" granules of Purves and Griesbach (7). The numbers of thyroidectomy cells in different grafts could not be well correlated with their TSH function.

Although acidophiles were not primary objects of this study, some of their changes were easily observable, especially in material stained with AF-trichrome. In grafts under the median eminence they were large, and their replenished cytoplasm had a distinctive, olive-green coloration (Fig. 16). This was not true for the grafts of the Ky and TL groups.

Epithelial cysts were observed in a few grafts of this group. The cysts usually had the form of small follicles lined with columnar epithelium and filled with intensely stained colloid (Fig. 8).

Grafts under the temporal lobe of the brain. The one 24-hour specimen in this group exhibited features very similar to those of a 24-hour graft under the median eminence. However, in the long-term experiments there was neither a functional nor a cytologic recovery. At the time of autopsy, these grafts were found adherent, at least in part, to the overlying brain and were in general well vascularized. Their size was somewhat less than that of the grafts in the median eminence groups. They were always composed of small acidophiles and chromophobes, among which only very occasional "basophiles" could be found (Fig. 7). No large gonadotrophs were ever seen in the grafts of this group.

DISCUSSION

The reported study was undertaken to determine whether the functional and cytologic losses in pars distalis grafts placed away from the hypothalamus can be caused merely by the operative trauma and the adverse effects of transplantation *per se* or whether they are caused specifically by the fact that the gland is removed from a special and direct hypothalamic influence. We have seen that the operative trauma indeed has a very damaging effect on the graft, reflected in the fact that extensive loss of parenchyma was noted in 24-hour grafts both under the kidney capsule and under the brain. However, the inflicted damage was not permanent and during the next few days there was remarkable parenchymal restoration.

Parallel consideration of grafts in different locations makes evident certain differences in changes occurring with time. Whereas the graft under the kidney capsule starts as a healthy gland, the graft under the median eminence stems from a gland already severely altered. Whereas, in both instances, an immediate, profound central loss of tissue was observed at

24 hours, the re-transplantation resulted in damage far more severe than did the initial transplantation. Only a few cells remained unimpaired. Although the graft on the kidney improved markedly in its histological organization in the days following transplantation, with apparent increase of mass of healthy parenchyma, the glandular cells became rapidly reduced in size and there was a progressive loss of "basophile" cell types. By contrast, although at 24 hours the grafts under the median eminence seemed to have been irreparably damaged, there was a surprising and rapid improvement in glands that remained there longer, improvement expressed not only in the form of increased mass and improved tissue organization, but also in reappearance of hypertrophied "basophiles." Grafts re-transplanted under the temporal lobe, apparently damaged to a degree equal to that in glands placed under the median eminence, failed to show either the functional or the cytological recovery observed in the latter. In general, then, while grafts under the renal capsule seemed functionally quiescent and uniform in cell composition, grafts under the median eminence gave the impression of vigorous secretory activity.

In addition to normal "basophiles," castration cells were characteristic of grafts under the median eminence from non-cycling animals or irregularly cycling ones. Since the work of Hohlweg and Junkmann (8) it has been recognized that castration cells are not formed in grafts placed at a distance from the diencephalon. This was true in the present study, where these cells were seen only in grafts under the median eminence. In such an experiment, when the graft is first returned under the median eminence, the animal is virtually a castrate, for its ovaries will have atrophied while the graft has been on the kidney. One might, therefore, suppose that if the ovary proves refractory many of the first gonadotrophs become castration cells. Once the refractory ovary finally responds, however, their numbers would decrease. Small numbers of castration cells in the grafts of normally cycling, fertile animals may have been left over from that earlier period. Yet it is well recognized that occasional castration cells are to be found in seemingly normal rats. It is puzzling that in spite of the abundance of castration cells and the implied hypersecretion of gonadotropin in the 4 anestrous rats of the median eminence groups, there was no significant degree of ovarian stimulation. Their grafts were as large, as well placed and as well vascularized as the other twenty.

The status of thyrotrophs and the respective numbers of thyroidectomy cells in grafts of the various groups could not be well related in individual cases to the functional status measured by thyroid uptake of I^{131}. But the generalization is true that there were considerably greater numbers of large thyrotrophs and thyroidectomy cells in grafts under the median eminence than in grafts under the temporal lobe or on the kidney. Their presence in reduced numbers in grafts on the kidney fits in general with the low grade TSH secretion indicated there.

In the normal gland there is a characteristic localization of thyrotrophs, LH-secreting gonadotrophs and FSH-secreting gonadotrophs, according to Purves and Griesbach (5). In the grafts under the median eminence, apart from the fact that the respective types are frequently distributed in clusters, one cannot state that the original pattern is retained. It was also not possible to distinguish 2 kinds of gonadotrophs (9) unless the observed dark and light staining cells represented them.

If LH-secreting cells are confined to the interior of the normal pars distalis, one would be forced to conclude that they were destroyed by the original massive central infarction and arose *de novo* in the re-transplanted gland. The origins of the various cell types, in fact, present an interesting problem for the future. Do they all arise anew from primitive stem cells or are they present in the kidney grafts in reduced size and often degranulated? If so, they may merely enlarge and become charged with secretion when stimulated by the hypothalamus. These questions apply not only to recognized normal cell types but to the less usual elements represented by the epithelial cysts that were found in several of the grafts under the median eminence. A systematic study of grafts under the median eminence for varying short intervals should be instructive.

It was seen that the operative trauma did indeed cause immediate, severe damage to the grafts. This fact in itself might have been sufficient to explain the loss of function and cellular differentiation, were it not for the results obtained following re-transplantation. The adverse effects of that operation were so severe that had we been aware of them in advance it is doubtful that this experiment would have been attempted. Happily, this was not known, and it can now be said that in spite of two successive episodes of severe trauma some local influence of the hypothalamic region restores not only relatively normal function but a relatively normal cell picture as well in grafts re-transplanted under the diencephalon. This evidence strongly supports the opinion that to have normal function, morphology and staining characteristics, the cells of the pars distalis must have direct access to stimuli from the hypothalamus, stimuli which are presumably chemical in nature and transmitted by the hypophysial portal vessels.

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ORGANIZING ACTION OF PRENATALLY ADMINISTERED TESTOSTERONE PROPIONATE ON THE TISSUES MEDIATING MATING BEHAVIOR IN THE FEMALE GUINEA PIG[1]

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ABSTRACT

The sexual behavior of male and female guinea pigs from mothers receiving testosterone propionate during most of pregnancy was studied after the attainment of adulthood. As a part of the investigation, the responsiveness of the females to estradiol benzoate and progesterone and to testosterone propionate was determined.

The larger quantities of testosterone propionate produced hermaphrodites having external genitalia indistinguishable macroscopically from those of newborn males. Gonadectomized animals of this type were used for tests of their responsiveness to estradiol benzoate and progesterone and to testosterone propionate. The capacity to display lordosis following administration of estrogen and progesterone was greatly reduced. Male-like mounting behavior, on the other hand, was displayed by many of these animals even when lordosis could not be elicited. Suppression of the capacity for displaying lordosis was achieved with a quantity of androgen less than that required for masculinization of the external genitalia.

The hermaphrodites receiving testosterone propionate as adults displayed an amount of mounting behavior which approached that displayed by the castrated injected males receiving the same hormone.

The data are uniform in demonstrating that an androgen administered prenatally has an organizing action on the tissues mediating mating behavior in the sense of producing a responsiveness to exogenous hormones which differs from that of normal adult females.

No structural abnormalities were apparent in the male siblings and their behavior was essentially normal.

The results are believed to justify the conclusion that the prenatal period is a time when fetal morphogenic substances have an organizing or "differentiating" action on the neural tissues mediating mating behavior. During adulthood the hormones are activational.

Attention is directed to the parallel nature of the relationship, on the one hand, between androgens and the differentiation of the genital tracts, and on the other, between androgens and the organization of the neural tissues destined to mediate mating behavior in the adult.

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INVESTIGATORS interested in reproductive behavior have demonstrated that one role of the gonadal hormones in adult male and female mammals is to bring to expression the patterns of behavior previously organized or determined by genetical and experiential factors (1, 2, 3, 4, 5). The hypothesis that these hormones have an organizing action in the sense of patterning the responses an individual gives to such substances has long been rejected (6, 7, 8, 9, 10). As far as the adult is concerned, this conclusion seems well founded. Female hormone, instead of feminizing castrated male rats as Kun (11) claimed, increased their activity as males (6). Male and female guinea pigs gonadectomized the day of birth, and a female rat with a congenital absence of the ovaries, displayed normal patterns of behavior when injected with the appropriate hormones as adults (3, 9, 12).

Unexplored since the studies of Dantchakoff (13, 14, 15), Raynaud (16) and Wilson, Young and Hamilton (17), is the possibility that androgens or estrogens reaching animals during the prenatal period might have an organizing action that would be reflected by the character of adult sexual behavior. If the existence of such an action were revealed, it would 1) extend our knowledge of the role of the gonadal hormones in the regulation of sexual behavior by providing information bearing on the action of these hormones or related substances during the prenatal period, 2) be suggestive evidence that the relationship between the neural tissues mediating mating behavior and the morphogenic fetal hormones parallels that between the genital tissues and the same hormones, and 3) direct attention to a possible origin of behavioral differences between the sexes which is *ipso facto* important for psychologic and psychiatric theory (18). Although comprehensive experiments have not yet been performed, initial investigations with an androgen have yielded effects which are so much more in line with current thought in the area of gonadal hormones and sexual differentiation (19, 20, 21, 22) than the earlier experiments on behavior, that the results are summarized here.

MATERIALS AND METHODS

(the production of hermaphrodites)

Most of the experimental animals were born to mothers which had received intramuscular injections of testosterone propionate[2,3] during much of gestation. One group was composed of females in which there were no visible abnormalities of the external

[2] Testosterone propionate (Perandren propionate) was supplied by Ciba Pharmaceutical Products, Inc.

[3] The injections were made by Mr. Myron D. Tedford, a Public Health Service Predoctoral Fellow, who is using these and other animals treated similarly for a study of the structural changes in the gonads, genital tracts, and external genitalia, and the course of gestation. We are indebted to him for supplying us with the animals whose behavior was investigated.

genitalia. These are referred to as the *unmodified females*. Their mothers were given an initial injection of 1 mg. of testosterone propionate some time between day 10 and day 27 after conception and 1 mg. every third or fourth day thereafter until the end of pregnancy.

The larger group was composed of females in which the external genitalia at the time of birth were indistinguishable macroscopically from those of their male siblings and untreated males. These animals are designated *hermaphrodites*. Laparotomy was necessary in order to distinguish these genetical females from males; it was performed within the first week after birth. Their mothers received an initial injection of 5 mg. of testosterone propionate on day 10, 15, 18, or 24 of the gestation period and 1 mg. daily thereafter until day 68.

Control animals were females and males from untreated mothers from the same stock as the experimental animals.

All these animals, i.e. the unmodified females, the hermaphrodites, their male siblings, and the control females and males, were used in four experiments designed to test the effects of testosterone propionate received prenatally on the responsiveness of the animals as adults to male and female hormones.

EXPERIMENTAL

Experiment I. *The behavior of gonadectomized adult unmodified females and hermaphrodites injected with estradiol benzoate and progesterone.*

Subjects

Fourteen females from untreated mothers.
Fourteen unmodified females.
Nine hermaphrodites.
Eight males from untreated mothers.

Except for four unmodified females gonadectomized when they were 45 days old, all the unmodified females and hermaphrodites were gonadectomized at 80 to 150 days of age. No data from the laboratory indicate that the response to exogenously administered sex hormones is influenced by age at the time of gonadectomy. The eight males were castrated before they were 21 days old.

Tests

After gonadectomy, when the animals were 90 to 160 days old, tests were made of the responsiveness to 1.66, 3.32, and 6.64 μg. of subcutaneously injected estradiol benzoate followed 36 hours later by 0.2 mg. of progesterone.[4] Observations were continuous for 12 hours, beginning immediately after the injection of progesterone. Following the procedure of Goy and Young (4) hourly checks were made for the occurrence of the lordosis reflex in response to fingering. Individual records were kept of this measure of behavior and of the frequency of male-like mounting.

In three tests the control females, hermaphrodites, and males were ob-

[4] Estradiol benzoate (Progynon-B) and progesterone (Proluton) were supplied by the Schering Corporation.

served for the occurrence of mounting in the absence of exogenous hormone. The unmodified females were given one such test.

The means and medians of the measures of behavior for which data were obtained were calculated from the individual averages and they are based on the data from the animals which responded to the hormones. For purposes of statistical analysis, maximum values (12 hours) for latency and 0 values for all other measures were arbitrarily assigned to the individuals failing to respond.

Results

The data bearing on all the measures of the estrous response except mounting are summarized in Table 1. The lower values for the per cent of

TABLE 1. DURATION OF HEAT AND LORDOSIS IN GONADECTOMIZED GUINEA PIGS GIVEN DIFFERENT AMOUNTS OF ESTRADIOL AND 0.2 MG. OF PROGESTERONE

Subjects	Tests* N	Per cent of tests positive for estrus	Mean latency in hours	Mean duration of heat in hours	Median duration of max. lord. in seconds
		1.66 μg.			
Control females	19	89	5.7	5.7	11.5
Unmodified females	20	65	6.5	2.8	8.5
Hermaphrodites	9	22	8.5	2.5	2.0
Castrated males	8	38	6.0	1.2	2.0
		3.32 μg.			
Control females	33	94	4.4	7.3	12.3
Unmodified females	38	68	5.6	2.8	5.1
Hermaphrodites	18	22	8.0	2.0	3.0
Castrated males	16	31	4.5	3.2	2.7
		6.64 μg.			
Control females	28	96	3.7	7.2	9.3
Unmodified females	22	77	5.8	3.3	6.0
Hermaphrodites	18	22	9.2	2.0	2.0
Castrated males	16	0	—	—	—

* All the animals were given one or more tests at each level of hormone.

tests positive for estrus, the mean duration of heat, and the median duration of the maximum lordosis were conspicuous effects of the treatment given prenatally and the differences among the groups are highly significant ($P < .001$). Among the two groups of experimental females and the castrated males, the low gutteral growl which is so characteristically a part of the pattern of lordosis in normal females, was commonly, and in some individuals always, lacking. Had the estimation of the duration of maximum lordosis been based only on complete responses, the differences among the groups would have been even greater.

Variations in medians for the duration of maximum lordosis were not systematically related to quantity of estradiol given prior to the tests. The analysis, therefore, was based on the medians of individual averages over all dosages. These medians were 11.3, 6.5, 2.3, and 2.5 seconds for

control females, unmodified females, hermaphrodites, and castrated males, respectively. The median of the unmodified females, which most closely resembles that of the control females, is significantly different ($U = 22$, $P < .002$) from that of the controls.

Other differences also are indicative of the changes that were induced. Per cent response and duration of heat tended to increase in the control groups as the quantity of injected estradiol was increased. Latency which is related inversely to the duration of heat (4) decreased. Among the experimental groups (unmodified females, hermaphrodites, and castrated males), similar relationships were seen only in the unmodified females.

In general the suppression of the capacity to display lordosis was proportional to the quantity of androgen injected prenatally. Amounts in-

TABLE 2. THE QUANTITY OF MOUNTING WITH AND WITHOUT ESTRADIOL AND PROGESTERONE

Subjects	Without hormone	With hormone*
	Mean number of mounts	Mean number of mounts
Control females	0	10.7
Unmodified females	0	8.8
Hermaphrodites	4.4	5.6
Castrated males	11.8	16.7

* Variation in the amount of mounting was not related to the quantity of estradiol. The means therefore are based on the averages for each individual whether the dosage was 1.66, 3.32, or 6.64 μg. of the hormone.

sufficient to alter external genital structures resulted in disturbances in the lordosis in only 50% of the animals, but the larger amounts that produced the hermaphrodites affected the lordosis in all. Within each group the effect on lordosis was not related to the quantity of androgen received prenatally. Among unmodified females, even siblings differed, one showing complete suppression of the lordosis and the other responding normally. The findings demonstrate that suppression of the capacity for displaying lordosis does not depend on masculinization of the external genitalia; clearly less androgen was required for the former than for the latter.

Additional evidence for the masculinizing effect of the prenatally administered androgen is provided by the data on the male-like mounting displayed by each group (Table 2). When estradiol and progesterone were injected all groups displayed mounting, and the differences among the groups are not statistically significant. In contrast, on tests when no hormones were given, the hermaphrodites and castrated males were the only animals that mounted.

The interval from the beginning of the test to the display of mounting differed among the groups. Of the males which mounted, all did so at least once during the first hour. Of the 7 hermaphrodites which mounted, 5 or

71% mounted at least once during the first hour, but only 1 normal female (7%) and 1 unmodified female (7%) mounted this early in the test. The modal time for the onset of mounting was the 1st hour for the castrated males and hermaphrodites and the 6th and 7th hours for the control females and unmodified females, respectively. In this respect the hermaphrodites closely resembled the castrated males and seem to have been masculinized. The latency of mounting in the unmodified females was not different from that in the control females.

In one way the mounting performance of the unmodified females did differ from that of the controls. More unmodified than control females displayed mounting on tests after injections when the lordosis reflex could not be obtained. Of 8 unmodified females which failed to show lordosis, 6 or 75% mounted. Because of the small number of control females which failed to display lordosis after injection, older data on normal females from the same genetical stock are used for comparison. These data combined with those from the present study reveal that of 38 normal females failing to display lordosis after injection with comparable amounts of estradiol and progesterone only 4 or 10.5% mounted. The difference between the proportions of control females and unmodified females displaying mounting in the absence of lordosis is significant (C.R. = 4.02, $P < .001$). Inasmuch as mounting was displayed spontaneously by the hermaphrodites, it was not possible with the animals available to determine the extent to which this behavior was being shown in response to the estradiol and progesterone.

Conclusions

1. Prenatally administered testosterone propionate suppressed the capacity for displaying lordosis following gonadectomy and the injection of estradiol and progesterone. The effect was manifested either by an absence of lordosis or by a marked abnormality in its character when it was displayed.

2. Suppression of the capacity for displaying lordosis was achieved with a smaller quantity of the androgen than was necessary for the gross modification of the external genitalia.

3. The capacity to display male-like mounting was not suppressed.

4. Quantities of testosterone propionate sufficient to suppress lordosis and masculinize the genitalia also reduced the interval before mounting behavior was displayed.

Experiment II. *Permanence of the effects of prenatally administered androgen.*

Subjects

Group 1. Three hermaphrodites used in the previous experiment.

Group 2. Seven unmodified females used in the previous experiment.

Group 3. Eight control females used in the previous experiment.

Group 4. Six hermaphrodites injected with 500 μg. of testosterone propionate per 100 gm. body weight per day from birth to 80 days of age.

Group 5. Six normal females injected with the same amount of testosterone propionate from birth to 80 days of age.

Group 6. Five mothers of hermaphrodites injected with testosterone propionate during pregnancy as described in Materials and Methods.

Group 7. Eight untreated females comparable in age with those injected with testosterone propionate during pregnancy.

The animals in Groups 1 through 5 were gonadectomized when they were 80 to 150 days of age, those in Groups 6 and 7 when they were 1.5 to 3 years old. The operations on the animals in Group 6 were performed approximately 10 months after the last injection of testosterone propionate.

Tests

All the animals received 3.32 μg. of estradiol benzoate followed 36 hours later with 0.2 mg. of progesterone. The tests were similar to those given the hermaphrodites, unmodified females, and controls in Experiment I. The number, however, differed for each group and is shown in the description of the results. The values reported in the tables and the statistical treatment of the data were determined by the methods described in Experiment I.

Results

The behavior of the 3 hermaphrodites, the 7 unmodified females, and the 8 control females is summarized in Table 3 and compared with that dis-

TABLE 3. BEHAVIORAL RESPONSES TO 3.32 μG. OF ESTRADIOL AND 0.2 MG. OF PROGESTERONE

		Tests at 6–9 months of age	Tests at 11–12 months of age
Hermaphrodites (Group 1)	Per cent response	33.0	0
	Latency to heat in hours	7.5	—
	Duration of heat in hours	2.5	—
	Median maximum lordosis in seconds	2.0	—
	Mean number of mounts	3.0	45.2
Unmodified females (Group 2)	Per cent response	55.0	71.0
	Latency to heat in hours	6.3	7.5
	Duration of heat in hours	2.2	2.3
	Median maximum lordosis in seconds	4.0	5.8
	Mean number of mounts	8.7	17.5
Normal females (Group 3)	Per cent response	95.0	94.0
	Latency to heat in hours	4.4	6.1
	Duration of heat in hours	7.2	4.5
	Median maximum lordosis in seconds	10.0	10.2
	Mean number of mounts	9.9	9.6

TABLE 4. PER CENT RESPONSE, DURATION OF HEAT, AND MAXIMUM LORDOSIS AFTER CESSATION OF TREATMENT WITH TESTOSTERONE PROPIONATE FROM BIRTH TO 80 DAYS OF AGE

		Approximate age in days at time of test			
		90	140	160	175
Hermaphrodites (Group 4)	Per cent response	0	0	0	0
	Mean duration of heat in hours	—	—	—	—
	Median maximum lordosis in seconds	—	—	—	—
Females (Group 5)	Per cent response	0	84	66	66
	Mean duration of heat in hours	0	4.6	1.7	3.7
	Median maximum lordosis in seconds	0	9.0	5.5	9.5

played during the earlier tests when the animals were 6 months old. The results reported in Table 3 are based on at least 2 tests of each individual at each age level. No significant change occurred in the hermaphrodites and unmodified females for per cent response, latency to heat, duration of heat, and the duration of maximum lordosis. The normal females, however, showed a significant decrease in the duration of heat ($T=0$, $P=.01$), reflecting perhaps a decrease in responsiveness to the hormones as the animals aged. The increase in mounting is significant for the unmodified females ($T=0$, $P=.02$). The 3 hermaphrodites displayed increased mounting behavior, but the increase could not be evaluated statistically. Of the normal females, 3 showed increases, 3 a decrease, and 2 remained the same.

The contrast between the effects of prenatal and postnatal treatment is revealed by the results obtained from the animals treated neonatally (Groups 4 and 5) and from those treated during pregnancy (Group 6). During the period after withdrawal of the testosterone propionate, 5 of the 6 normal females which had been injected for 80 days after birth regained the ability to display lordosis, whereas the hermaphrodites did not (Table 4). The effects of the postnatally administered androgen on the mounting behavior displayed by the animals in the two groups were complex and their presentation is being postponed until a further discussion can be given. The females treated with testosterone propionate while pregnant (Group 6) did not, like their "daughters," lose the capacity to display lordosis. Comparison of their behavior in response to estradiol and progesterone in five tests with that of untreated females of the same age (Group 7) (Table 5), revealed that the differences between the groups are not significant for latency, duration of heat, and mounting.

TABLE 5. BEHAVIOR OF NORMAL FEMALES TREATED WITH TESTOSTERONE PROPIONATE FOR 5 DAYS DURING PREGNANCY AND TESTED 10 MONTHS LATER

	Per cent response	Latency of heat in hours	Duration of heat in hours	Mean no. of mounts
Treated females (Group 6)	84	6.7	4.2	17.8
Untreated females (Group 7)	62	7.6	3.2	8.1

Conclusions

1. The suppression of the capacity for displaying the feminine components of the sexual behavior pattern which followed the administration of testosterone propionate prenatally appears to have been permanent.

2. Amounts of testosterone propionate which were effective prenatally had no conspicuous lasting effects when administered postnatally.

Experiment III. *The behavior of gonadectomized hermaphrodites in response to testosterone propionate.*

Subjects

Five hermaphrodites gonadectomized between 86 and 112 days of age.
Five normal females gonadectomized between 80 and 106 days of age.
Eight normal males castrated before 21 days of age.

When the animals were approximately 180 days old all received 2.5 mg. of testosterone propionate daily for 16 consecutive days.

Tests

A sexual behavior test was given the day before the first injection. Additional tests were given on days 1 and 2 of the injection period, and every other day thereafter until each animal had received 9 tests. The ninth test was given the day of the sixteenth injection.

Results

The median value for mounting by the hermaphrodites and females in the single test prior to the injection of testosterone propionate was 0. For the males the median was 5.5.

The remaining data are summarized in Table 6. They demonstrate the masculinizing effect of prenatally administered testosterone propionate on the female. Castrated males and hermaphrodites obtained the highest sexual behavior scores, the control females the lowest. The overall difference in scores was significant ($P \sim .02$). The differences between the castrated males and hermaphrodites were not significant, whereas both groups differed significantly from the control females ($P = .05$). The overall difference in the number of tests to the first display of mounting was significant ($P < .01$). As with the sexual behavior scores, the difference between males

TABLE 6. MASCULINE BEHAVIOR IN GONADECTOMIZED ADULT ANIMALS INJECTED WITH TESTOSTERONE PROPIONATE

Group	Mean sexual behavior score	Mean mounts per test	Median number of tests to the first display of mounting	Median mg. of t.p. prior to the display of mounting
Spayed untreated females	2.1	5.8	7.0	30.0
Spayed hermaphrodites	3.6	15.4	3.0	10.0
Males castrated prepuberally	5.0	20.5	1.5	3.8

and hermaphrodites was not significant, but both groups differed significantly from the control females ($P = .02$). There was a significant overall difference ($P < .01$) in the amount of testosterone propionate required before the first appearance of mounting. Again, the hermaphrodites resembled the castrated males in that there was no significant difference between these two groups, but both groups displayed mounting with significantly less hormone ($P = .02$) than the control females.

Conclusions

1. Adult hermaphrodites gonadectomized and injected with testosterone propionate were more responsive to this hormone than gonadectomized normal females.
2. The earlier appearance and greater strength of masculine behavior by the hermaphrodites given testosterone propionate are believed to be effects of the prenatally administered testosterone propionate on the tissues mediating masculine behavior and therefore to be expressions of its organizing action.

Experiment IV. *The behavior of adult male siblings of the hermaphrodites.*

Subjects

Five males from untreated mothers.

Five males born to mothers receiving testosterone propionate during pregnancy. No hormone was administered after birth.

Five males born to mothers receiving testosterone propionate during pregnancy. These animals received 500 μg. of the hormone per 100 gm. body weight daily beginning 1 to 3 days after birth and continuing 80 to 90 days.

Tests

Five tests were given when the animals were 11 months old. In a test the subject was placed with a receptive female of approximately the same size, and the frequency of the display of selected measures of behavior was recorded for a maximum of 10 minutes. These measures included sniffing and nibbling, nuzzling, abortive mounting, mounting, intromissions, and ejaculation. A description of the measures and the method for computing scores are given by Valenstein, Riss and Young (23).

Results

The mean scores are summarized in Table 7. It is clear that any effect of the exogenous testosterone propionate was slight. There was no evidence of suppression of the capacity to display masculine behavior, if anything, the animals receiving the hormone prenatally achieved higher scores than the controls.

TABLE 7. MEAN SEXUAL BEHAVIOR SCORES OBTAINED BY THE THREE GROUPS OF ADULT MALES

Groups*	Tests				
	I	II	III	IV	V
Untreated	6.9	6.6	9.2	7.2	10.4
Testosterone propionate prenatally	10.4	9.3	9.1	9.3	12.2
Testosterone propionate prenatally and postnatally	10.9	11.2	7.3	11.1	9.4

* Difference among the groups not significant; $F = 1.30$; $df = 2, 12$.

Conclusion

The sexual behavior of adult males which had received testosterone propionate prenatally was not significantly different from that of untreated controls.

DISCUSSION

The data from the four experiments summarized in the preceding sections support the hypothesis that androgenic substances received prenatally have an organizing action on the tissues mediating mating behavior in the sense of altering permanently the responses females normally give as adults. This possibility was suggested by the work of Dantchakoff (13, 14, 15), Raynaud (16), and Wilson, Young and Hamilton (17). Probably, however, because interest in the role of gonadal hormones in the regulation of mating behavior was concentrated so largely on the neonatal individual and adult, the suggestion was never incorporated in our theories of hormonal action. This step may now be taken, but when what has been learned from the present investigation is related to what has long been known with respect to the action of androgens on the genital tracts, a concept much broader than that suggested by the older studies is revealed.

The embryonic and fetal periods, when the genital tracts are exposed to the influence of as yet unidentified morphogenic substances (19, 20, 21, 22, 24), are periods of differentiation. The adult period, when the genital tracts are target organs of the gonadal hormones, is a period of functional response as measured by cyclic growth, secretion, and motility. The response depends on whether Müllerian or Wolffian duct derivatives have developed, and although generally specific for hormones of the corresponding sex, it is not completely specific (25). For the neural tissues mediating mating behavior, corresponding relationships seem to exist. The embryonic and fetal periods are periods of organization or "differentiation" in the direction of masculinization or feminization. Adulthood, when gonadal hormones are being secreted, is a period of activation; neural tissues are the target organs and mating behavior is brought to expression. Like the geni-

tal tracts, the neural tissues mediating mating behavior respond to androgens or to estrogens depending on the sex of the individual, but again the specificity is not complete (26, 27).

An extension of this analogy is suggested by the work done on the embryonic differentiation of the genital tracts, particularly that by Burns and Jost and summarized in their reviews (20, 21, 22). It will be recalled from the data reported in the present study that testosterone propionate administered prenatally affected the behavior of the male but slightly, whereas the effects on the female were profound. Not only was there a heightened responsiveness to the male hormone as revealed by the stronger masculine behavior displayed when testosterone propionate was given, but there was a suppression of the capacity to display the feminine components in response to treatment with an estrogen and progesterone. In studies of the genital tracts there were no effects on the male except for a slight acceleration in the development of the prostate and seminal vesicle and an increase in the size of the penis (28). Within the female, the Wolffian duct system was stimulated (13, 14, 15, 28, 29), and locally, when a fetal testis was implanted into a female fetus (20, 21), there was an interruption of the Müllerian duct on that side. What has not been seen when an exogenous androgen was administered, except by Greene and Ivy (30) in some of their rats, is a suppression or inhibition of the Müllerian duct system corresponding to the suppression of the capacity for displaying the feminine component of behavior.

The failure to detect a corresponding suppressing action on the Müllerian duct does not exclude the possibilities 1) that such an effect will be found, and 2) that the suppressing action is in the nature of a reduction in the responsiveness of the genital tract to estrogens rather than in the inhibition of its development. Such an effect was encountered in rats given testosterone propionate prenatally (17) when it was found that uterine as well as behavioral responses to estrogen and progesterone were suppressed.

A final suggestion with respect to the analogy we have postulated arises from a comparison of our results with those reported by Dantchakoff and Raynaud. These investigators stressed the increased responsiveness of their masculinized guinea pigs and mice to exogenous androgens, and seemed to regard the change as the expression of an inherent bisexuality. The possibility that there might have been a suppression of the capacity to respond as females and therefore an inequality of potential does not seem to have been considered. Like Dantchakoff (13, 14, 15), Raynaud (16), and many others (9, 31, 32, 33, 34, 35), the existence of a bisexuality is assumed. We suggest, however, that in the adult this bisexuality is unequal in the neural tissues as it is in the case of the genital tissues. The capacity exists for giving behavioral responses of the opposite sex, but it is variable and in most mammals that have been studied and in many lower vertebrates

as well, it is elicited only with difficulty (27). Structurally, the situation is similar. Vestiges of the genital tracts of the opposite sex persist and are responsive to gonadal hormones (36, 37), but except perhaps in rare instances, equivalence of organs and responses in a single individual is not seen (36, 37, 38, 39).

The concept of a correspondence between the action of gonadal hormones on genital tissues and neural tissues contains much that is new and its full scope is not yet clear. The possibility must be considered that the masculinity or femininity of an animal's behavior beyond that which is purely sexual has developed in response to certain hormonal substances within the embryo and fetus.

Thus far the permanence of the effect achieved when testosterone propionate was received prenatally has not been achieved when the same hormones were administered to adults or to newborn individuals. The dependence of this "permanence" on the action of the hormone during a possible critical period must be ascertained.

The nature of the modifications produced by prenatally administered testosterone propionate on the tissues mediating mating behavior and on the genital tract is challenging. Embryologists interested in the latter have looked for a structural retardation of the Müllerian duct derivatives culminating in their absence, except perhaps for vestigial structures found in any normal male. Neurologists or psychologists interested in the effects of the androgen on neural tissues would hardly think of alterations so drastic. Instead, a more subtle change reflected in function rather than in visible structure would be presumed.

Involved in this suggestion is the view that behavior may be treated as a dependent variable and therefore that we may speak of shaping the behavior by hormone administration just as the psychologist speaks of shaping behavior by manipulating the external environment. An assumption seldom made explicit is that modification of behavior follows an alteration in the structure or function of the neural correlates of the behavior. We are assuming that testosterone or some metabolite acts on those central nervous tissues in which patterns of sexual behavior are organized. We are not prepared to suggest whether the site of action is general or localized.

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QUANTITATIVE RESPONSE OF THE RAT OVARY TO PITUITARY GONADOTROPIN AS MODIFIED BY ESTROGEN[1]

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ABSTRACT

A purified pituitary gonadotropin containing predominantly FSH activity was administered in graded doses to hypophysectomized and intact immature female rats, with and without concurrent administration of diethylstilbestrol in large doses.

A more striking and consistent ovarian weight increase to pituitary gonadotropin, brought about through stimulation of early ovarian follicle development, regulation of further follicular progression, and depression of interstitial tissue overgrowth was observed in the diethylstilbestrol-treated hypophysectomized animals.

LARGE doses of estrogen given to the hypophysectomized immature rat potentiate ovarian follicular development and subsequent luteinization while tending to inhibit the response of ovarian interstitial tissue to various gonadotropins (1). Quantitative aspects of this phenomenon in both the intact and hypophysectomized immature rat are presented.

METHODS

Immature rats of the Holtzman strain 22–24 days of age were used. Hypophysectomy was performed by the parapharyngeal technique. Diethylstilbestrol in sesame oil at a dose of 1 mg. daily was employed as the estrogen. A purified sheep pituitary gonadotropin, which contained predominantly FSH activity was used (Gonadophysin, Searle).[2] This material was dissolved in 0.9% saline for administration. Daily subcutaneous injections were given for 4 days beginning immediately after hypophysectomy, or at 22–24 days of age in the intact rats, and animals were sacrificed 48 hours after the last injection. The ovaries were then quickly dissected free of adventitious tissue, weighed to the closest 0.05 mg. and fixed in 10% buffered formalin. Histologic sections were stained with H & E. The incidence of solid follicles (S.F.), vesicular follicles (V.F.), corpora lutea (C.L.), and cicatrized corpora lutea (C.C.L.), was recorded on a 4-point scale based on a gross estimate of the relative contribution of these elements to the total ovarian mass.

RESULTS

The ovarian weight response to the pituitary gonadotropin in ascending doses described a sigmoid curve which could be roughly divided into 3

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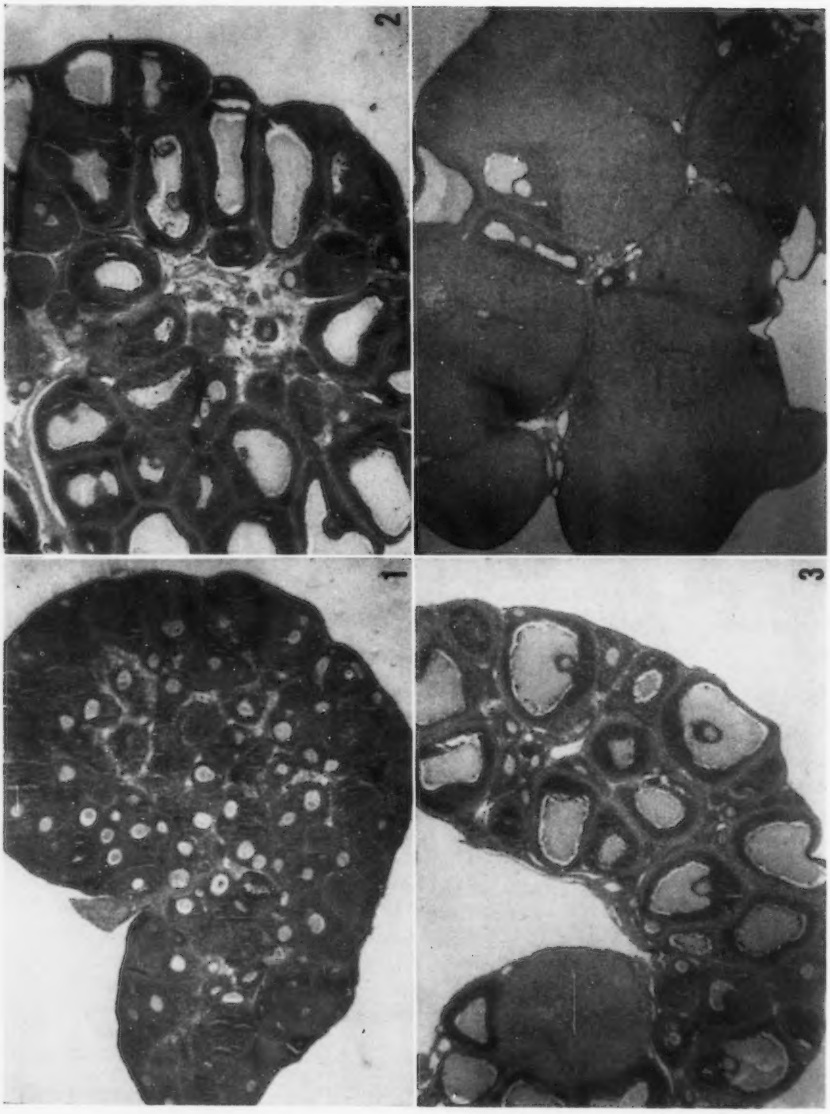

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succeeding phases. With the smaller doses of gonadotropin, a relatively shallow dose response curve was observed which correlated with follicle development to the stage of early antrum formation. The succeeding steep response curve noted with larger doses of gonadotropin could be ascribed to rapid development of vesicular (Graafian) follicles and subsequent corpus luteum formation. Further increase in gonadotropin dosage produced a plateau, followed by an erratic effect on ovarian weight due to superimposed processes of degeneration and cicatrization of formed corpora lutea. These events were more uniformaly produced in the hypophysectomized rat than in her intact counterpart (Tables 1 and 2).

Diethylstilbestrol stimulated the ovaries to the point of medium-sized solid follicle formation while depressing interstitial tissue elements. In the hypophysectomized immature rat, ovarian antrum formation could not be induced by estrogen alone. However, when graded doses of pituitary gonadotropin were administered concurrently with estrogen, a considerable potentiation of ovarian follicular development conducive to luteinization was observed. Similar effects were observed in both intact and hypophysectomized, immature rats, but they were potentiated and more uniform in the hypophysectomized animals.

Ovarian weight responses to increasing doses of pituitary gonadotropin administered to estrogen-treated and non-estrogen-treated hypophysectomized animals are compared in Table 2. The relative tardiness of early follicular development in non-estrogen-treated animals is evident (Fig. 1); this is in contrast to the more rapid and uniform development of ovarian follicles in the estrogen-synergized animals (Fig. 2). Estrogen potentiation continues to be apparent as the dose of gonadotropin is increased. At a daily dose of 0.25 mg. of gonadotropin, ovaries of non-estrogen-treated animals contain a variety of follicular stages with occasional corpus luteum formation (Fig. 3), while ovaries of estrogen-treated animals given the same dose of gonadotropin show uniformly advanced follicles with pro-

FIGS. 1–4. Ovaries of hypophysectomized immature rats treated with 4 daily doses of indicated materials beginning immediately after hypophysectomy, with autopsy 48 hours after last injection. All magnifications ×28.

FIG. 1. .08 mg. pituitary gonadotropin. Ovarian follicles in various stages of early solid follicle development.

FIG. 2. .08 mg. pituitary gonadotropin +1 mg. diethylstilbestrol. Relatively uniform, moderately advanced vesicular follicle formation is evident. Interstitial elements are not stimulated.

FIG. 3. .25 mg. pituitary gonadotropin. A wide variety of follicle types is seen with isolated corpus luteum formation. Cicatrization of large corpus luteum has begun. Stimulation of interstitial tissue is evident.

FIG. 4. .25 mg. pituitary gonadotropin +1 mg. diethylstilbestrol. A preponderance of young corpora lutea is evident. The 3 mature Graafian follicles still present are nearly ready for luteinization. One corpus luteum has begun involution.

TABLE 1. EFFECTS OF PITUITARY GONADOTROPIN WITH AND WITHOUT DIETHYLSTILBESTROL ON OVARIES OF INTACT IMMATURE RATS

Daily dose gonadotropin[1] (mg.)	+DES 1 mg./day				No DES			
	No. animals	Mean corrected ovarian wt. ± S.E.* (mg./100 g. body wt.)		Ovarian† components	No. animals	Mean corrected Ovarian wt. ± S.E.		Ovarian† components
Control	11	68.73	5.60	SF +++ VF +	7	23.41	0.64	SF +++ VF ++
.05	3	82.47	21.13	VF +++ CL ++	3	43.41	5.32	VF ++ CL +
.08	10	82.21	13.43	CL + CCL +	10	36.90	1.45	CL ++ CCL ++
.10	3	80.33	13.50	CL ++ CCL ++	3	60.36	12.63	CL ++ CCL ++
.15	13	118.87	9.30	CL + CCL +	12	53.18	3.79	CL ++ CCL ++
.20	3	124.33	13.32	CL +++ CCL ++	3	62.43	1.51	CL + CCL +
.22	10	192.62	11.84	CL ++ CCL ++	12	57.20	2.21	VF +++
.25	3	135.87	12.88	CL +++ CCL +++	3	125.80	15.56	CL +++ CCL +++
.30	14	128.12	16.75	CL ++ CCL ++	3	93.16	20.81	CL ++ CCL ++
.35	3	213.46	37.02	CL +++ CCL +++	3	93.63	15.29	CL +++ CCL +++
.40	3	198.66	5.49	CL ++++ CCL ++++	3	104.93	8.76	CL +++ CCL +++
.50					3	110.10	13.80	CL ++ CCL ++

[1] Doses administered 4 days, beginning on day of hypophysectomy.
* Standard Error.
† SF = solid follicles, VF = vesicular follicles, CL = corpora lutea, and CCL = cicatrized corpora lutea; + = <10%, ++ = 10–25%, +++ = 25–50%, and ++++ = >50%, of ovarian mass.

gression to nearly complete luteinization (Fig. 4). As the dosage of gonadotropin is further increased and involution of corpora lutea occurs, ovarian weight response becomes increasingly erratic—even in the presence of estrogen. The relatively weak interstitial-cell-stimulating activity present in the gonadotropin becomes evident with the largest doses, as manifested by increased interstitial tissue as well as premature (aberrant) luteinization of follicles.

DISCUSSION

Hypophysectomized rats develop inertia in their ovarian follicular response to pituitary gonadotropin even when treatment is started immediately after operation. This lag is abundantly compensated by concurrent administration of estrogen in substantial doses. Though the present study has been conducted with diethylstilbestrol there is little doubt that the observed effect is a property common to many estrogenic compounds (1).

Estrogen potentiation of pituitary gonadotropin results from stimulation of the follicle in its early phases as well as depression of restricting interstitial tissue. Estrogens similarly modify the intrinsic pituitary gona-

TABLE 2. EFFECTS OF A PITUITARY GONADOTROPIN WITH AND WITHOUT DIETHYLSTILBESTROL ON OVARIES OF HYPOPHYSECTOMIZED IMMATURE RATS

Daily dose gonadotropin[1] (mg.)	+DES 1 mg./day				No DES			
	No. animals	Mean corrected ovarian wt. ± S.E.* (mg./100 g. body wt.)		Ovarian† components	No. animals	Mean corrected ovarian wt. ± S.E.		Ovarian† components
Control	10	58.08	2.96	SF ++++	15	17.86	1.88	SF +
.05	16	65.73	3.00	VF +				
.08	10	67.32	4.36	VF +	9	22.38	0.83	SF +++ VF +
.10	24	78.84	2.82	VF ++	5	24.22	1.27	SF +++ VF +
.15	29	120.46	8.03	VF +++	13	29.16	2.20	SF ++ VF ++
.20	18	167.20	14.87	VF +++ CL ++	5	41.68	5.92	VF +++
.22	17	176.01	9.24	CL +++ CCL +	10	60.27	3.76	VF +++ CL + CCL +
.25	18	203.26	12.57	CL ++++ CCL ++	11	90.08	9.24	VF ++++
.30	19	207.39	12.19	CL ++++ CCL +++	15	79.90	7.57	VF ++++ CL + CCL +
.35	4	219.00	22.74	CL ++++ CCL +++	5	118.14	13.18	CL ++ CCL ++
.40	12	224.10	12.41	CL ++++ CCL ++++	14	101.06	10.14	CL ++ CCL ++
.50	18	201.72	12.03	CL ++++ CCL ++++	10	110.76	7.54	CL +++ CCL +++
.60					5	116.30	13.70	CL +++ CCL +++
.70					5	115.80	13.86	CL +++ CCL +
1.00	11	202.47	8.67	CL ++++ CCL ++++				

[1] Doses administered 4 days, beginning on day of hypophysectomy.
* Standard Error
† SF = solid follicles, VF = vesicular follicles, CL = corpora lutea, and CCL = cicatrized corpora lutea; + = <10%, ++ = 10–25%, +++ = 25–50%, and ++++ = >50%, of ovarian mass.

dotropins of intact animals. In contrast, the ovarian weight gain induced by pituitary gonadotropin in combination with human chorionic gonadotropin (3, 4) or pituitary ICSH (5) results from ovarian interstitial tissue proliferation, which is of less physiologic interest.

It appears that the effects observed in estrogen-gonadotropin treated animals are largely produced by pituitary follicle stimulating hormone (FSH), though it would seem proper at the present state of our knowledge to assign to this factor the further role of corpora lutea production from ripe Graafian follicles. With large doses of the gonadotropin studied, sufficient interstitial cell stimulating hormone activity was present to partially overcome the weak antagonism to interstitial tissue formation provided by the estrogen. This response has been previously studied in detail by administering combinations of human chorionic gonadotropin and pituitary gonadotropin to immature hypophysectomized rats with and without estrogen (1).

The effects of several "purified" FSH and ICSH (LH) preparations have been studied in estrogen-treated hypophysectomized rats (6). All have shown, with ascending doses, the described sequence of events, with luteinization occurring as the proper stage of follicular development is reached (6). The effects of large doses of ICSH, however, became manifest by interstitial tissue proliferation and premature luteinization which inhibited rather than augmented final ovarian weight (1).

The present method of studying the dose-response to pituitary FSH appears to hold promise as an assay procedure based on quantitative gain in ovarian weight paralleled by follicular development and subsequent luteinization via a relatively physiologic route.

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ASSAY OF FOLLICLE-STIMULATING HORMONE IN THE HYPOPHYSECTOMIZED ESTROGEN-TREATED IMMATURE FEMALE RAT[1]

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ABSTRACT

An assay for pituitary follicle-stimulating hormone (FSH) using the estrogen-treated, hypophysectomized immature rat is presented.

Linear dose-response curves, which correlated with histological evidence of maturation of follicles, were observed with various preparations tested for FSH activity. Crude pituitary tissue from several species, as well as purified pituitary gonadotropins, were assayed for FSH content; potencies were evaluated by comparison of the slopes of the regression lines produced.

THOUGH several methods for the assay of pituitary follicle stimulating hormone (FSH) using the immature female rat have been described (1, 2, 3, 4), the lack of pure preparations of FSH and incomplete knowledge of its full physiologic effects on the ovary have hampered the development of meaningful assay methods for this hormone.

The present assay for FSH employing the diethylstilbestrol-treated hypophysectomized immature rat has developed from studies concerned with the interaction of estrogens and gonadotropins on the ovaries (5, 6, 7). Efforts were directed toward utilizing those factors which would provide a quantitative increase in ovarian weight brought about by a correlated sequence of ovarian follicle development, ovulation, and corpus luteum formation.

Comparisons of desiccated crude pituitary tissue obtained from several species, and of purified gonadotropins characterized by other assay techniques, have been made using the present method.

METHODS

Assay rats were 22 to 24 day old females of the Holtzman strain. Injections by the subcutaneous route were begun immediately after hypophysectomy. Each animal was treated with 1 mg. of diethylstilbestrol in 0.1 ml. of sesame oil given at a separate site from the material under study. Fresh-frozen pituitary glands from the various species tested were defatted and desiccated in acetone and administered on the basis of dried

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weight. Gonadotropins were dissolved or suspended in 0.9% saline and injected in a volume of from 0.1 to 0.5 ml. Control animals were treated with diethylstilbestrol alone. Injections were administered daily for 4 days with autopsy 48 hours after the final injection. Animals were killed by cervical fracture and the ovaries from each animal quickly removed, dissected free of adventitious tissue, weighed together, and preserved in 10% buffered formalin. Ovarian weights were corrected to a final body weight of 100 gm. Histologic preparation of the ovaries was by the standard H & E technique. The microscopic characteristics of ovarian tissue were summarized on the basis of relative contribution to the ovarian volume of solid follicles, vesicular follicles and corpora lutea. The frequency of older corpora lutea as manifested by the appearance of cicatrization was also recorded. A rough appraisal of interstitial cell-stimulating hormone (ICSH) activity was based on histologic evidence of repair and proliferation of ovarian interstitial tissue as well as the occurrence of premature luteinization (7).

Each gonadotropin was tested at a minimum of 2 dose levels, using a total of 388 animals for the 13 preparations assayed.

STATISTICAL ANALYSIS

Because the variance of the responses (ovarian weights) increased proportionately to the square of their mean at the various dosage levels, the responses were all converted to logarithms. Thus, dose was plotted against log response as recommended by Finney (8). Inspection then suggested that this procedure removed the correlation between the mean of the log response and the variance within the log response, as was intended.

The model used for analysis was:

$$Y = \mu + \beta_1 X_1 + \beta_2 X_2 + \cdots + \beta_{13} X_{13} + \alpha_1 + \alpha_2 + \cdots + \alpha_{13} + \delta_1 X_1^2 + \cdots + \delta_7 X_7^2 + \gamma_1 X_1^3 + \gamma_2 X_2^3 + \gamma_3 X_3^3 + \theta_1 X_1^4 + \theta_2 X_2^4 + \phi_1 X_1^5 + \epsilon_1$$

where:

Y = Log response (log corrected weight of ovary)

μ = Over-all Y -intercept of experiments

β_i = Linear regression coefficient of ith drug

α_i = Adjusted effect of the log response at zero dosage δ_i, γ_i, θ_i and ϕ_i = quadratic, cubic, quartic and quintic regression coefficients respectively.

ϵ_1 = Sampling error (between observations within each dosage level)

Under the assumption of linearity, the lack of fit estimated from the combined quadratic, cubic, quartic and quintic components was accepted as the best estimate of experimental error.

Due to the nature of the experimental design herein employed, it was felt that the sampling error $\hat{\sigma}_{\epsilon_1}^2$ was in all likelihood underestimated because most of the experiments at a single dosage level were carried out simultaneously, and at a time different from those at other dosage levels. On the other hand, it is recognized that the experimental error $\hat{\sigma}_{\epsilon_2}^2$ is probably slightly over-estimated, due to lack of absolute linearity. For purposes of the F test, however, it was felt more desirable to use the larger, rather than the smaller, estimate of error.

Least squares estimates of these various coefficients required the solution of thirty-nine normal equations under the restriction $\sum \alpha_i = 0$. (There were 13 agents tested at from 2 to 6 dose levels.) Gonadophysin was tested at 6 levels and used as the standard against which the other 12 preparations were compared. The equations were arranged in the proper order so that the analysis of variance could be obtained on the IBM 650 using an abbreviated Doolittle program (9). By assuming a linear log response the regression coefficients necessary for obtaining the relative potencies were estimated by

TABLE 1. COMPARATIVE ASSAYS OF SEVERAL GONADOTROPINS

Pituitary preparation	Levels tested	R_i in %+S.E.	Regression coefficients (b values)	Dose range (mg.)	Number animals
Gonadophysin	6	—	0.021969	0.08– .25	115
Rat	5	48.68± 5.80	0.010695	0.1 – 0.5	39
Horse	4	3.65± 0.33	0.000801	1.0 –10.0	33
Pig	3	3.18± 0.30	0.000698	3.0 –10.0	37
Sheep	3	2.69± 0.21	0.000592	3.0 –10	61
Whale	3	2.27± 0.33	0.000499	3.0 –10	20
Rx 179-172 (Parke, Davis)	3	16.27± 6.29	0.003575	0.1 – 1.0	8
K 45308R (Armour)	2	46.25±12.95	0.010161	0.1 – 0.3	14
S 131A (McShan)	2	90.89±14.95	0.019967	0.1 – 0.3	9
X 5766 (Parke, Davis)	2	147.95±42.00	0.032503	0.03– 0.1	15
227-80 (Armour)	2	16.84± 5.55	0.003700	0.3 – 1.0	6
Beef	2	0.72± 0.14	0.000159	10.0 –20.0	24
Human	2	23.94±11.73	0.005259	0.5 – 1.5	7

solution of the 13 normal equations which came from the multiple regression model:

$$Y = \mu + \sum_{i=1}^{13} \beta_i X_i + \epsilon_2$$

where:

Y = log response

μ = Over-all Y intercept of all experiments

β_i = linear regression coefficients

X_i = dosage levels of the ith drug in mg./day

ϵ_2 = experimental error (lack of fit)

Results of the analysis of variance so carried out were as follows:

Source	*df*		*SS*	*MS*	*F*
Total	388		22.131909	—	—
Dosages		38	14.916972	0.39251	
Linear		13	13.237902	1.018300	17.04[2]
Intersection (after adjustment for linearity)		12	0.902077	0.075173	1.26
Experimental Error ($\hat{\sigma}_{\epsilon_2}^2$) (lack of fit)		13	0.776993	0.059769	
Sampling Error ($\hat{\sigma}_{\epsilon_1}^2$)	350		7.215157	0.020615	

The relative potency of each agent, with its standard deviation and its estimated value, as compared to the standard agent (Gonadophysin):

$$R_i = \frac{b_i}{b_1} \times 100,$$

is listed in Table 1. The S. E. of R_i was computed by generalizing the formula used by Steelman and Pohley (4).

RESULTS

Characteristics of the response of the hypophysectomized immature rat ovary to estrogen and to estrogen-gonadotropin stimulation have been previously described (7). In general, a linear dose response to gonadotropins of pituitary origin was observed. Assays of desiccated pituitary glands from various species were compared for gonadotropin content. Of

[2] The F value for linearity under this analysis is highy significant and that for intersection is not. This is as one wishes it to be for the bioassay technique here employed.

those species tested, rat pituitary tissue obtained from young adult male animals exhibited the highest content of FSH, followed in descending order by human, horse, pig (anterior pituitary), sheep, whale and beef pituitary. (Table 1). A crude fish pituitary powder—*Ictiobus cyprinellus* (Buffalo), exhibited no FSH activity at the maximum doses tolerated by the assay animals (15 mg.).

Several purified pituitary gonadotropins which had been quantitated by other assays for FSH and ICSH activity in the laboratories of their origin were assayed by the present technique. The preparations described as containing predominantly FSH activity proved most effective with the present assay (Table 1). Preparations which were reported to exhibit largely ICSH activity (LH 118, McShan, S 291, McShan and 2105F56, Parke, Davis) produced no response with the present technique at doses of 1 mg. daily. However, a possible exception occurred with preparation 227–80 (Armour), a material exhibiting strong ICSH activity but reported to contain only about 5% FSH (4). This preparation was shown by the present assay to have a somewhat high FSH content (Table 1).

The progression of ovarian follicular development, as observed microscopically, could be correlated with ovarian weight increases produced by the various preparations tested. A detailed description of this sequence of events has been described for one of the test substances (11). In general, corpus luteum formation occurred at an ovarian weight approximately double that of the estrogen-treated controls. There was, however, a tendency for less active materials to produce luteinization at a slightly lower ovarian weight.

The presence of ICSH was evident with relatively large doses of some of the preparations (human, rat, horse, 227–80 (Armour), Rx 179–172 (Parke, Davis), and Gonadophysin (Searle) as manifested by stimulation of interstitial cells, evidence of premature luteinization and slight hindrance of physiologic luteinization.

Minimally effective doses of FSH, while producing only an uncertain increase in ovarian weight, could be detected histologically by the presence of early vesicular changes in the otherwise characteristic medium-sized solid "estrogen" follicles. Excessive doses of FSH produced erratic responses but could be roughly quantitated by the incidence and degree of cicatrization of corpora lutea. These observations were of considerable value in adjusting further doses to a more useful range.

DISCUSSION

The assay technique described has been extensively used in this laboratory to follow the fractionation of pituitary gonadotropins. It has proven itself relatively sensitive, dependable, and reproducible. Though

the assay is, in all probability, largely a measure of FSH, a rough estimate of ICSH activity, at least when it is present in large amounts, can also be provided. However, the large doses of estrogen used generally tend to suppress the effects of small doses of ICSH (7).

The quantitation of each gonadotropic preparation is, in some regards, a series of physiologic studies. The poorly understood factors of antagonism and synergism, even of seemingly non-hormonal materials with gonadotropins (3, 10), is a constant source of confusion in the interpretation of strictly quantitative results. The opportunity to correlate potency of gonadotropins with the physiologic stages of ovulation and luteinization is thus considered of great importance.

It is of interest that assays for FSH using human chorionic gonadotropin as the augmenting factor (4) provide ovarian weight gains of the same order as those seen with the described technique, but by a considerably different mechanism, namely the overgrowth of interstitial tissue.

The present assay, like that of Steelman and Pohley (4), is informative when performed in intact animals; however the dose response, while of similar magnitude to that seen in the hypophysectomized animal, is comparatively erratic (11).

For assay in the optimal range 3 animals at 2 dose levels has been found to provide a useful estimation of FSH activity. Somewhat greater precision can be obtained by using 8–10 animals per dose, though no useful purpose appears to be served by further increasing the number of animals at each dose level.

While the present method obviously still falls short of an ideal assay technique for FSH, it is offered with the hope of providing semi-quantitative data and physiologic information for the fractionation and characterization of gonadotropic hormones.

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THE EFFECT OF GROWTH HORMONE ON THE *IN VITRO* INCORPORATION OF LEUCINE-2-C^{14} INTO THE PROTEIN OF RAT DIAPHRAGM[1]

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ABSTRACT

The *in vitro* incorporation of DL-leucine-2-C^{14} into diaphragm protein was investigated. Hypophysectomy decreases and growth hormone stimulates leucine-C^{14} incorporation in fed and fasted animals. The stimulation of leucine-C^{14} incorporation into diaphragm protein effected by growth hormone was not accompanied by a concomitant increase in Qo_2. Thyroidectomy, while reducing diaphragm Qo_2, did not alter leucine-C^{14} incorporation.

THE *in vivo* incorporation of isotopically labeled amino acids into protein has been utilized in several studies (1–5) designed to investigate the action of growth hormone on the retention of nitrogen by tissues. In such studies labeled amino acids were injected several minutes to several hours before the tissues were removed for the determination of the extent of labeling in tissue protein or various cytoplasmic fractions. After hypophysectomy the incorporation of amino acids into tissue protein was reduced (1–5), but the administration of growth hormone did not always restore the incorporation to normal (1, 3, 5). Lee and Williams (2) reported an increase in cystine-S^{35} incorporation in liver, kidney, heart and spleen following treatment of fed hypophysectomized rats with growth hormone. Friedberg and Greenberg (1) found an increase in the incorporation of methionine-S^{35} into skeletal muscle protein of hypophysectomized rats after growth hormone injections, but they noted that growth hormone treatment resulted in a reduction in amino acid uptake by the liver. Similarly, Reid *et al.* (3) found a decrease in leucine-C^{14} incorporation by liver cytoplasmic fractions of limit-fed hypophysectomized rats given growth hormone, while Korner (4) observed that liver cell fractions of such animals showed an increased incorporation of labeled amino acid.

The present study was undertaken to find a simple, reliable and versatile system which could be utilized to investigate, in detail, the influence of growth hormone on the transfer of amino acids into tissue protein. An *in vitro* system was adopted consisting of rat diaphragm incubated in a

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buffered medium containing labeled leucine. Similar systems have been employed by others (6–8) in studies of the effects of insulin on amino acid incorporation by muscle.

METHODS

In most of the following experiments, female rats of the Charles River strain weighing approximately 60 gm. were used. Larger rats (200–350 gm.) of both sexes were employed in some of the early experiments, but the specific activity of the diaphragm protein was considerably lower than that observed in diaphragms from the smaller animals. For this reason, only young animals were used subsequently. Young hypophysectomized animals were allowed to recover from the operation for at least one week before an experiment. The larger rats, however, were not used in experiments until two months after hypophysectomy. Thyroidectomy was performed two months before the rats were employed in experiments. During the second month following thyroidectomy, they were maintained on a diet containing 0.1% propylthiouracil to insure the functional deactivation of possible thyroid remnants.

Bovine growth hormone (NIH-BGH-1)[3] was administered intraperitoneally once a day at a dose of 1 mg. per rat for periods of 4 to 6 days.

Rats were sacrificed by a blow on the neck, and the diaphragm was quickly excised and placed in freshly gassed buffer. It was then divided into two hemidiaphragms. Each of these was blotted and placed immediately into a chilled Warburg flask containing 2.0–3.0 ml. Krebs-Ringer phosphate buffer, pH 7.4. The buffer contained DL-leucine-2-C^{14} (Tracerlab, Inc., S.A. 0.51 mc./mM) at a concentration of 0.1 mM (25,000 C.P.M./ml. medium) and, in most cases, glucose at a concentration of 200 mg.%. If oxygen consumption measurements were to be made concomitantly, 0.2 ml. 10% KOH was placed in the flask centerwell. The flasks were then equilibrated to 37° C for 10 minutes during which they were gassed with 100% oxygen, and then incubated with shaking for periods up to 90 minutes. Usually, one of a pair of hemidiaphragms was incubated for 30 minutes and the other for 60 minutes. The incorporation reaction was terminated by rapidly chilling the flasks to 0° C.

Previous *in vitro* studies (c.f. 8) demonstrated that glucose (in the absence of added insulin) impeded the incorporation of alanine into the protein of diaphragms of normal fed rats. The incorporation of leucine into normal rat diaphragms, however, was not affected (8). Control experiments designed to establish the effects of glucose on the *in vitro* incorporation of leucine into diaphragms of hypophysectomized rats were performed (to be published), and it was observed that glucose was without effect on this system (13).

To prepare the protein for counting, the diaphragms were homogenized in chilled distilled water and an equal volume of 8% trichloracetic acid (TCA) was added to precipitate the protein. The precipitated protein was washed successively in 4% TCA containing 10 mg. DL-leucine-C^{12}/ml., 4% TCA (80° C), 2:2:1 ether: alcohol: chloroform (45° C), and acetone (45° C). After drying thoroughly, the protein was homogenized in acetone and plated out on discs of filter paper, which were dried and weighed. A Nuclear-Chicago gas flow counter with a Micromil end-window was used to count the samples. The radioactivity measurements were corrected for background and self-absorption and are expressed as counts/minute/mg. of protein.

RESULTS

Hemidiaphragms of fed hypophysectomized rats were found to incorpo-

[3] The bovine growth hormone was a gift of the Endocrinology Study Section of the National Institutes of Health.

rate significantly less ($P < 0.01$) leucine-2-C^{14} than similar muscles of normal rats, during an hour incubation period (Fig. 1). The administration of growth hormone to hypophysectomized rats essentially restored the decreased amino acid incorporation by the diaphragms to normal.

Although the data in Figure 1 were compiled from experiments on young rats (60 gm.), similar experiments were performed on larger animals

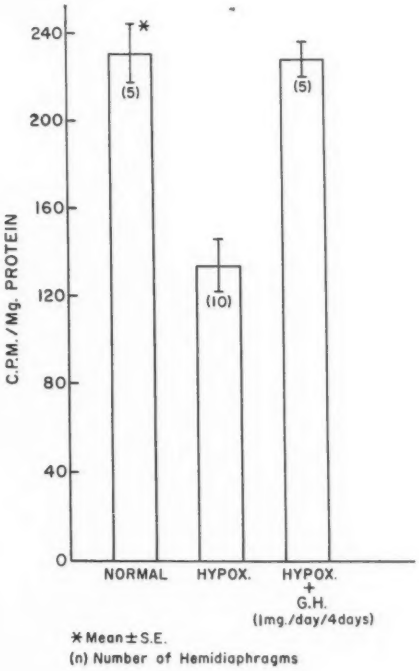

FIG. 1. The effects of hypophysectomy and growth hormone treatment on the incorporation of DL-leucine-2-C^{14} into the protein of hemidiaphragms of fed rats incubated for 60 minutes at 37° C in oxygenated buffer containing 200 mg. % glucose and DL-leucine-2-C^{14} (25,000 C.P.M./ml. medium) at a concentration of 0.1 mM.

(200 gm.) which had been hypophysectomized for a much longer time (two months). Normal rats of equivalent size were used as controls. The hemidiaphragms of these larger rats responded, in a like manner, with a decrease in incorporation of leucine after hypophysectomy, and an increase in incorporation following growth hormone treatment. The specific activity of the protein from these muscles, however, was lower and the magnitude of the responses was reduced. The reduced rate of leucine incorporation by the muscles of these larger rats can probably be explained, in part at least, by recent findings (9) which indicate that the rate of uptake of amino acids by rat skeletal muscle is inversely proportional to body weight.

Concomitant measurements of oxygen consumption made in the above experiments revealed that the Qo_2 of the hemidiaphragms of hypophysectomized rats (6.28 ± 0.28) was considerably lower than that of the normal

controls (9.26 ± 0.61). The reduced Qo_2 effected by hypophysectomy was not changed by growth hormone treatment (5.57 ± 0.29).

In some of the early experiments, the incubation flasks were equilibrated in air rather than gassed with 100% oxygen. Hemidiaphragms that were incubated in air had a much lower rate of amino acid incorporation than muscles incubated in oxygen (Fig. 2). However, the effect of growth hormone treatment was still fully evident ($P < 0.01$) (Fig. 2).

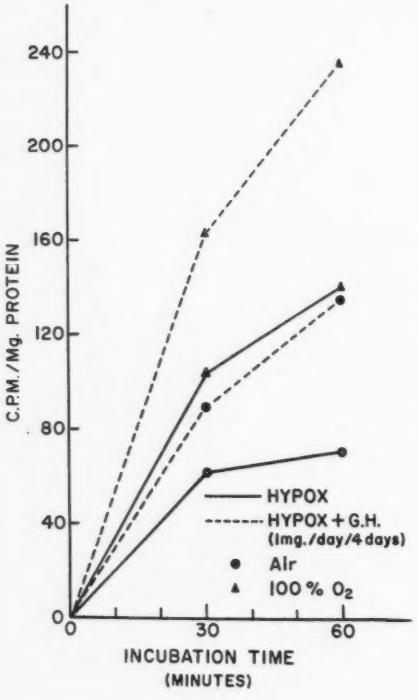

FIG. 2. The effects of incubation in air or 100% oxygen on the incorporation of DL-leucine-2-C^{14} into hemidiaphragm protein of fed hypophysectomized and growth hormone treated rats. Incubation was carried out at 37° C in buffer containing 200 mg. % glucose and DL-leucine-2-C^{14} (25,000 C.P.M./ml. medium) at a concentration of 0.1 mM.

The effect of fasting (24 hours) on the growth hormone induced stimulation of leucine incorporation by hemidiaphragms of hypophysectomized rats was investigated. The results in Figure 3 indicate that fasting did not influence the ability of growth hormone to stimulate the incorporation of leucine ($P < 0.01$), but did depress the rate of incorporation by approximately 20% in both the treated and control groups.

The effect of growth hormone on normal rats (200 gm.) was also studied. In the few experiments performed, growth hormone treatment doubled the rate of gain in body weight, but the incorporation of leucine by the diaphragms was not significantly altered.

Finally the effect of thyroidectomy on incorporation rate was investi-

gated. The hemidiaphragms of thyroidectomized rats (250–350 gm.) were found to have a leucine incorporation rate similar to the normal controls of equivalent size (Fig. 4), while, as expected (10), the oxygen consumption of the tissues was significantly reduced (Normal Control $Qo_2 = 4.74 \pm 0.13$; Thyroidectomized $Qo_2 = 4.05 \pm 0.13$; $P < 0.01$).

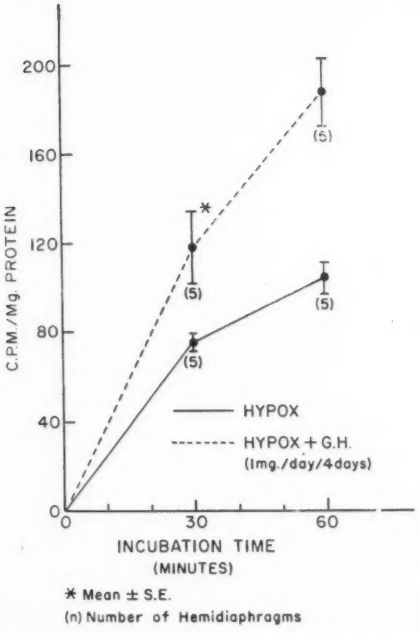

FIG. 3. The effect of growth hormone treatment of the incorporation of DL-leucine-2-C^{14} into the protein of hemidiaphragms of fasted (24 hours) hypophysectomized rats. Incubation was carried out at 37° C in oxygenated buffer containing 200 mg. % glucose and DL-leucine-2-C^{14} (25,000 C.P.M./ml. medium) at a concentration of 0.1 mM.

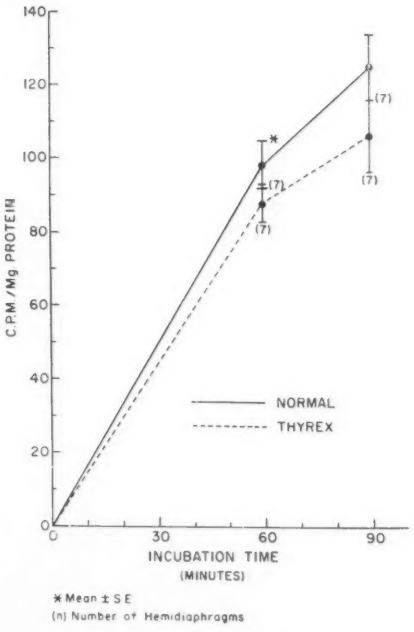

FIG. 4. The effect of thyroidectomy on the incorporation of DL-leucine-2-C^{14} into the protein of hemidiaphragms of fed rats incubated at 37° C in oxygenated buffer containing 200 mg. % glucose and DL-leucine-2-C^{14} (25,000 C.P.M./ml. medium) at a concentration of 0.1 mM.

DISCUSSION

The above experiments indicate that hypophysectomy decreases and growth hormone stimulates the transfer of labeled leucine into the protein of skeletal muscle *in vitro*. This effect can be demonstrated both in the fed and fasted state. The decrease in labeled amino acid incorportion following hypophysectomy occurred in muscles from small rats hypophysectomized for brief periods as well as in muscles from much larger animals hypo-

physectomized for two months. This decrease in incorporation would appear to result principally from a lack of growth hormone, since growth hormone given to hypophysectomized rats restored the decreased incorporation to normal. Removal of the thyroid from large rats failed to alter the incorporation of labeled leucine into diaphragm protein, suggesting again that the decreased incorporation seen in hypophysectomized animals is due to the absence of growth hormone rather than to hypothyroidism.

From the information obtained in these experiments, it is impossible to arrive at any conclusions regarding the manner in which hypophysectomy decreases and growth hormone increases the incorporation of leucine-C^{14} into muscle protein. It is possible that the intracellular leucine pool may be larger than normal in the muscle cells of hypophysectomized rats or that these cells release leucine into the medium during incubation at a greater than normal rate. Either or both of these factors could have produced a dilution of the isotope in the system resulting in the observed effects. The extent to which these elements contributed to the experimental findings remains to be determined. However, there are other reasonable explanations for the observed effects. Available evidence suggests that insulin promotes the transport of amino acids into muscle cells (7–8), and it is also conceivable that growth hormone, by itself or in conjunction with insulin, may exert a specific effect on amino acid transport. The experiments of Noall *et al.* (11) concerning the acute administration of growth hormone on the concentration of the nonutilizable amino acid, α-aminoisobutyric acid, by various tissues would support this possibility, since this substance was concentrated to a somewhat greater extent after an injection of growth hormone. However, the stimulation by growth hormone of the incorporation of amino acids into protein probably involves more than the amino acid transport system of the cell. Korner (12) using a cell free system, where intracellular pool size or cell membranes cease to be factors, has found that the impairment in the ability of the rat liver microsome to incorporate labeled leucine which occurs after hypophysectomy appears to reside in the microsomal protein itself rather than in the activating or intermediate steps involved in the incorporation of amino acids into protein.

If the effects observed in the present study were indeed due to alterations in any or all of the several steps in the protein synthetic mechanism, then it is of considerable interest that, within the experimental conditions employed, the effect of growth hormone on labeled amino acid incorporation into muscle protein can be demonstrated in the absence of a measurable change in oxygen consumption of the tissue. Growth hormone stimulated leucine-C^{14} incorporation by muscles of hypophysectomized rats without altering oxygen consumption. Conversely, the low oxygen tension of the medium bathing the muscles incubated in air did not influence the pronounced stimulatory effect of growth hormone on amino acid in-

corporation. Moreover, the depression in oxygen consumption of the diaphragm occasioned by thyroidectomy was without effect on the leucine-C^{14} incorporation by this tissue. These considerations would suggest then that the primary action of growth hormone on the incorporation of amino acids into protein is not mediated through the oxidative energy generating system of the muscle cell.

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ON THE METABOLISM OF 3,3′-DIIODOTHYRONINE AND 3,3′,5′-TRIIODOTHYRONINE

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ABSTRACT

1. After injection of 3,3′-diiodothyronine to thyroidectomized rats, the presence of 3,3′-diiodothyroacetic acid has been demonstrated in the kidney.
2. After injection of 3,3′,5′-triiodothyronine to thyroidectomized rats, the presence of 3,3′,5′-triiodothyroacetic acid and of minute amounts of 3,3′-diiodothyroacetic acid has been shown in the kidney. 3,3′-diiodothyronine is also present and 3,3′-diiodothyroacetic acid is probably a metabolic product of it.
3. As the administration of both isomers of triiodothyronine (3,3′,5′ and 3,5,3′) leads to the formation of 3,3′-diiodothyronine, the deiodination process of these substances appears to be relatively unspecific.

ALTHOUGH the metabolism of L-thyroxine (T_4) and 3,5,3′-triiodothyronine (T_3) has been extensively studied, the two more recently identified thyroid hormones, 3,3′,5′-triiodothyronine (T'_3) and 3,3′-diiodothyronine (T'_2), have received little attention as yet. It has been shown, however, that these two substances injected in physiological doses are concentrated by the liver and the kidneys with some formation of glycuroconjugates and with deiodination (1). No information on the degradation of their carbonic skeleton or on the mechanism of dehalogenation is available. Since T_4 and T_3 are chiefly transformed into their thyroacetic derivatives (2, 3), 3,5,3′,5′-tetraiodothyroacetic acid (T_4A) and 3,5,3′-triiodothyroacetic acid (T_3A), and to a slight extent deiodinated to form T_3(2, 3) and T'_2 (4) respectively, this study was undertaken to determine whether similar metabolism pathways exist for T'_2 and T'_3.

MATERIALS AND METHODS

For T_4 and T_3, the kidney appears to be the best material to study the occurrence of metabolic derivatives of T'_3 and T'_2. Thus, we injected T'_3 and T'_2, labelled on the 3′-carbon atom prepared by iodination of 3-monoiodo-L-thyronine (5) to male rats (180–200 gm.), thyroidectomized one month previously, and killed the animals from 1 to 3 hours later. Analysis of labelled constituents of the kidneys consisted of extraction and separation by chromatography and chromatophoresis on paper. Pure synthetic iodothyroacetic acids (6) and hormones (5) labelled in 3′ or 3′,5′ have been used as carrier and reference substances. Reference substances are localized on chromatograms and

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electrophoregrams by ceric sulfate-arsenious-acid reaction (7) and radioactive derivatives by autography.

Preparation of kidney extracts. Groups of six or seven rats received 0.5μg.T′$_2$ ((36μc-I^{131}) or T′$_3$ (28μcI^{131}) in three injections of an identical amount of hormone, on three successive hours. Animals were killed by bleeding 1 hour later in the case of T′$_3$, 3 hours later in the case of T′$_2$. The kidneys were dissected, frozen in dry ice and ground to a fine powder. This was extracted by 1% NH_4OH until all the radioactivity of the tissues (1.5% of the injected I^{131} was removed. Extracts were lyophylized and the dry residue was extracted five times with a mixture of peroxide-free ether (95%) and absolute alcohol (5%); less than 1% of radioactivity was lost. Water was added to the lipid free powder (7 ml. for the kidneys of six rats) and NH_4OH 0.5 N (34 ml.) was added drop by drop to dissolve it. The proteins were precipitated by 95% alcohol (25 ml.) and separated by centrifugation. After alcoholic extraction of the precipitate, 84% of the initial radioactivity was recovered in the solution. An equal volume of chloroform, with 10 ml. of 2N NH_4OH was added and the chloroform layer was separated by centrifugation and eliminated. All these operations were carried out at 0° C and under nitrogen.

Ammoniacal extracts were evaporated to 5 ml at a temperature below +30° C under reduced pressure, in a nitrogen atmosphere. After addition of five drops of concentrated NH_4OH, the solution was deposited in a thin line on no. 1 Whatman paper and eluted with *n*-butanol saturated with water. Pigments remain in the paper and the radioactive material is extracted. The eluate (solution 1) is ready for chromatography and electrophoresis.

RESULTS

A. *Experiments with 3,3′-diiodothyronine* (T′$_2$). Bidimensional radioautochromatogram of solution 1, obtained with suitable solvents is reproduced in Figure 1.

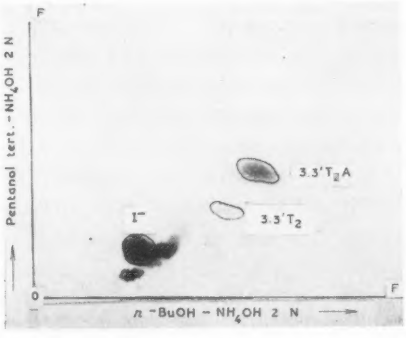

FIG. 1. Bidimensional Autoradiochromatogram (Whatman paper no. 1) of the kidney extract of thyroidectomized rats injected with labelled 3,3′-diiodothyronine. Solvents: 1) *n*-butanol saturated with 2N NH_4OH; 2) tertiary pentanol saturated with 2N NH_4OH. I^--iodide; 3,3′-T$_2$=3,3′-diiodothyronine, 3,3′-T′$_2$A =3,3′-diiodothyroacetic acid. No intermediary product of deiodination has been identified, but radioactive spots of unknown substances are present (Rf near the iodide spot).

This shows the presence of T′$_2$, of iodide and of T′$_2$A. To confirm the identity of the latter, solution 1 was purified by preparative electrophoresis. The radioelectrophoregram (Fig. 2) shows four radioactive bands, numbered 1, 2, 3, 4. These have been cut out separately and eluted with *n*-butanol saturated with water. Since the position of carrier T′$_2$A is identical with that of strip 2, this has been especially studied. Its eluate has been submitted to a preparative monodimensional chromatography with *n*-butanol saturated with 2N NH_4OH. A strip of the chromatogram has

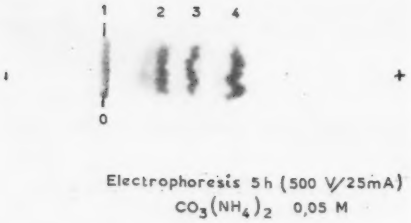

FIG. 2. Autoradioelectrophoregram of kidney extract of thyroidectomized rats injected with labelled 3,3′-diiodothyronine (500 volts, 25 mA, buffer: $(NH_4)_2CO_3$ 0.05 M, 5 hr., Whatman paper no. 1).

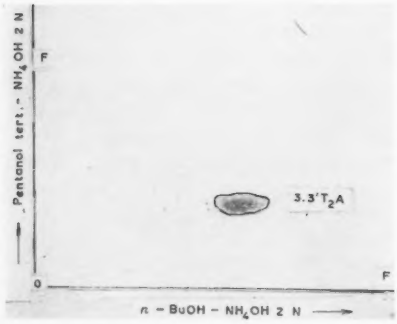

FIG. 3. Bidimensional autoradiochromatogram (Whatman paper no. 1) of purified eluate of strip 2 of figure 2. Solvent: 1) *n*-butanol saturated with 2N NH_4OH; 2) tertiary pentanol saturated with 2N NH_4OH; 3,3′-T′$_2$A = 3,3′-diiodothyroacetic acid. The presence of T′$_2$A in the kidneys of rats injected with T′$_2$ is thus demonstrated.

been cut at the level of the pure T′$_2$A carrier and eluted with *n*-butanol saturated with water. Bidimensional chromatography (Fig. 3) confirms the identification of T′$_2$A.

B. *Experiments with 3,3′,5′-triiodothyronine* (T′$_3$). Extracts prepared from kidneys as described above (solution 1) are submitted to chromatography and to chromatoelectrophoresis. Figures 4 and 5 show results obtained by these methods.

The identity of reference spots with those of radioactive material established the presence in kidney extracts of a mixture containing: a) iodide, b) minute amounts of unaltered T′$_3$, c) relatively important amounts of T′$_2$, d) small amounts of T′$_3$A, eventually T′$_2$A, e) two unknown substances X_1 and X_2. The identification of T′$_2$ as a deiodination product of T′$_3$ needs

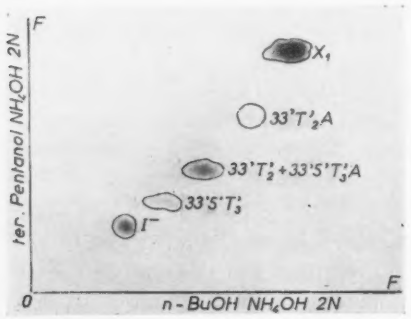

FIG. 4. Bidimensional autoradiochromatogram (Whatman paper no. 1) of the kidney extract of thyroidectomized rats injected with labelled 3,3′,5′-triiodothyronine. Solvents: 1) *n*-butanol saturated with 2N NH_4OH; 2) tertiary pentanol saturated with 2N NH_4OH. I^- = iodide; 3,3′,5′-T′$_3$ = 3,3′,5′-triiodothyronine; 3,3′-T$_2$′ = 3,3′-diiodothyronine; 3,3′,5′-T$_3$′A = 3,3′,5′-triiodothyroacetic acid; 3,3′-T′$_2$A = 3,3′-diiodothyroacetic acid; X_1 = unknown.

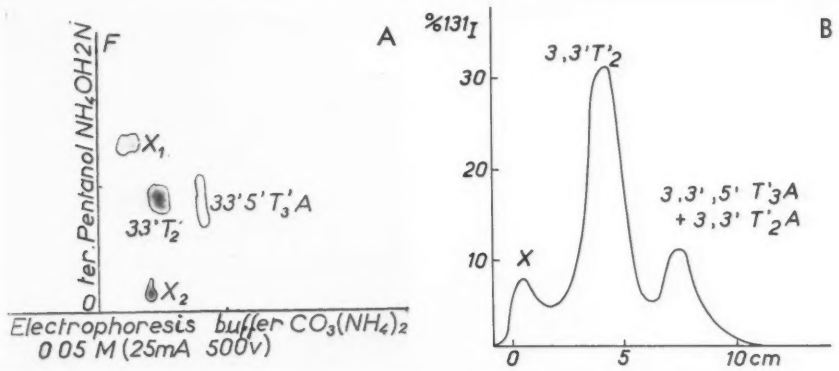

FIG. 5. A. Autoradiochromatoelectrophoregram of a fraction of the same extract as on figure 4: 1) chromatography with tertiary pentanol saturated with 2N NH_4OH; 2) electrophoresis in $(NH_4)_2CO_3$ 0.05 M (500v., 2 mA, 5 hours). Same symbols as in Fig. 1, X_1 and X_2 = unknown, iodide is not present on this figure owing to its high electrophoretic mobility.

B. Radioelectrophoregram (quantitative measurement of radioactivity along the electrophoregram) of a fraction of the same extract as for figure 4 in $(NH_4)_2CO_3$, 0.05 M (500 v., 2 mA, 5 hours). Same symbols as in figure 4, iodide eliminated owing to its high mobility.

no special confirmation because of the very definite agreement of the radioactive and reference spots, as in kidney extracts of animals injected with T_3 (3). That of T'_3A and T'_2A, not encountered in tissues before our work, needs confirmation.

Solution 1 has been purified by preparative electrophoresis (480 v, 25 mA, in $(NH_4)_2CO_3$ 0.05M, 5 hours). This separated the iodide (eliminated in figure 5 B owing to its large ionic mobility towards the anode) from three radioactive bands: unknown substances X_1 and X_2 compounds, T'_2 and a band of higher mobility, eventually yielding T'_3A and T'_2A. This strip has been cut and eluted with *n*-butanol saturated with water and the eluate submitted to chromatography and chromatoelectrophoresis in suitable solvents. Figure 6 A shows a spot of pure T'_3A isolated from a kidney extract by elution of the monodimensional chromatogram from which the curve of figure 6 B has been established. This shows the position of radioactivity along a chromatogram (solvent: tertiary amylol saturated with 2N NH_4OH) of the eluate of the third band of the electrophoregram corresponding to figure 5 B.

From these figures, the presence of T'_3A and of very minute amounts of T'_2A is beyond doubt.

DISCUSSION

It is of interest, on two major points, to compare our results with those of studies on the metabolism of T_3 and T_4 in the kidney (2, 3, 4), the forma-

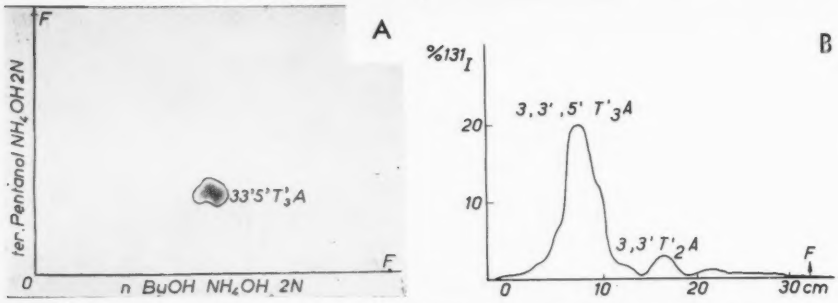

FIG. 6. A. Bidimensional autoradiochromatogram of a spot of 3,3′,5′-triiodothyroacetic acid (3,3′,5′-T'_3A) prepared from the renal extract of rats injected with T'_3 (see text) solvents: 1) *n*-butanol saturated with 2N NH_4OH, 2) tertiary pentanol saturated with 2N NH_4OH.

B. Autoradiochromatogram of a fraction of the eluate of the third strip of the electrophoregram used to established the curve of figure 5 B; solvent: tertiary amylol saturated with 2N NH_4OH; 3,3′-T'_2A = 3,3′-diiodothyroacetic acid; 3,3′,5′-triiodothyroacetic acid).

tion of iodothyroacetic derivatives and the deiodination of the hormones. The iodothyroacetic acids seem no longer to be the "active form" of the hormones, as hypothetically supposed in the previous year (7) but they are a very important step in one of the pathways of the degradation of all the iodothyronines. It is not possible to decide whether their presence in the kidney is due only to their concentration from plasma or due to the catabolic activity of renal tissue itself. Both processes are possibly responsible for it. In any case, it is likely that all the natural iodothyronines follow the fate of α-amino acids, through transamination or oxidation by L-amino-acidoxidase. The presence of T'_2 in kidney after injection of T'_3 must be pointed out, because the same derivative has also been found in the kidney of animals injected with T_3. This is very surprising for two reasons: the 3,5 and 3′,5′ positions of the iodine atoms seem to be very different in their reactivity toward chemical agents (8); the 3,5 positions are more labile to hydrogenation whereas the 3′,5′ positions of T_4 seem to be more rapidly deiodinated in the whole animal (9). This is not the case in the kidney however, where the iodine atom bound to the 3′-carbon in T'_3 and that bound to the 3-carbon T_3 react in the same way. These findings are in some respects complementary to recent observations (10, 11) showing that the labelled 3,5 and 3′,5′ positions of T_4 are not deiodinated preferentially *in vitro*.

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RADIOIODINE CONCENTRATION BY THE FETAL MOUSE THYROID

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ABSTRACT

Twenty-four hours following subcutaneous injection of radioiodine into pregnant mice various fetal and maternal tissues were removed, weighed and their radioactivity measured. Measurements at various days of gestation show the fetal mouse thyroid first concentrates iodine between the 14th and 15th days of gestation in mice with an average gestational period of 19 days. Development of the ability to concentrate iodine precedes by more than a day the formation of follicles and colloid in the gland.

As the iodine uptake of the litter increases greatly toward term, there is indication of a corresponding decrease in the iodine uptake by the maternal thyroid. Near term, the concentration of iodine in fetal thyroids and the total uptake by thyroids of whole litters equals or exceeds the concentration, in and uptake by, the maternal thyroid.

It is suggested that changes in the placenta in mass and in capacity to concentrate and transmit iodine, particularly between the 17th and 18th days, may affect the iodine content of fetal tissues.

THE onset and progressive development of function of the fetal thyroid gland has received considerable attention. In recent investigations of this subject, radioactive iodine has been used. Part of a dose of radioiodine administered to a pregnant mammal crosses the placenta and may be accumulated by the fetal thyroid. Measurements of the radioactivity of fetal glands following administration of radioiodine to pregnant females at various times in gestation shows the time in development when the fetal thyroid commences to function and the increasing avidity of the gland for iodine.

Speert, Quimby, and Werner (1) reported that the fetal mouse thyroid first accumulates radioiodine in fetuses from mothers injected with radioiodine on the 16th day of gestation (gestational period 20 days). Fetuses were removed 48 hours following administration of radioiodine to the mother and the radioactivity of *whole fetuses* measured with a Geiger counter.

We suspected that a small animal such as the mouse may actually be

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concentrating considerable amounts of radioiodine per unit mass of fetal thyroid tissue at earlier times in gestation (2). Younger thyroids are exceedingly small and though they may be very actively concentrating radioiodine, the total amount they could take up might be so little that the radioactivity of the fetus would be considered insignificant. This supposition was based on the following calculation from known data on rats. The volume of a rat thyroid is approximately 20 mm^3. The volume of the average thyroid cell is about 10^{-6} mm^3. Therefore the number of cells per thyroid is approximately 2×10^7. If 20 μc were administered to an adult rat with a 10% radioactive iodine uptake, 2 μc would appear in the thyroid 24 hours later. This is equivalent to 4.4×10^6 disintegrations per minute per gland; or .22 disintegrations per minute per thyroid cell. Therefore 1000 such cells would only yield counts equivalent to the average scintillation well-type counter background. If the entire embryo were counted, a slight avidity for iodine of the immature fetal thyroid could be missed, especially since some NaI^{131} would be distributed throughout the embryo, raising the background and negating any possibility of observing iodine uptake in a very small group of cells.

MATERIALS AND METHODS

In the present study, the fetal thyroid uptake was determined at various times in gestation by dissecting and weighing the fetal thyroids and then measuring the radioactivity in the glands themselves. By knowing the weights of the glands, it was possible to express the iodine uptake as μc. of I^{131} per mg. of wet thyroid tissue. Weighing, and measuring the radioactivity of the younger thyroids was facilitated by pooling the thyroids dissected from each litter.

Female white mice of a Swiss Bagg strain with a gestational period of 19 days were reared in a temperature controlled room in individual cages on distilled water and a specially prepared diet that was consistently low in iodine and that did not vary over the course of the experiment. Animals were placed on the special diet about 30 days prior to their use. Pregnancies were dated by limiting mating time and examining for vaginal plugs. At various stages of gestation (11 to 17 days) pregnant females were injected subcutaneously with 100 μc. of carrier-free NaI^{131}. Twenty-four hours following administration of the radioiodide, the fetuses were removed and weighed. The fetal thyroids were dissected out along with the adjacent larynx. Inclusion of the attached portion of the larynx was necessary to avoid the loss of radioactivity that could be demonstrated to result from direct handling of the thyroid. By examination and measurement of the block of tissue containing the fetal thyroid, both whole and in serial sections, it was possible to calculate very nearly the proportion of the tissue that was thyroid. These proportions were utilized in calculating the weights of the fetal thyroids. Thyroids of fetuses assayed on the 12th to 15th days were pooled with those of their littermates for weighing and the measurement of radioactivity. Older thyroids were treated individually. Pooled or individual thyroids were placed on pre-weighed pieces of aluminum foil and weighed to the nearest $\frac{1}{10}$ mg. This was accomplished as soon after dissection as possible. The thyroids were kept in a moist chamber until weighed. The thyroids plus the aluminum foil were digested in counting tubes containing NaOH. The radioactivity was measured in a Nuclear, DS-3, scintillation well-type counter, shielded to reduce background radiation to about 180 counts per minute.

Fetal liver and brain, placenta, and maternal thyroid, liver, blood, and kidney were removed, weighed, and their radioactivity measured. All calculations were corrected for decay and for the usual instrumental factors.

RESULTS

The fetal mouse thyroid begins to concentrate iodine between the 14th and 15th days of gestation (Fig. 1). In fetuses from mothers injected on the 14th day of gestation and assayed on the 15th, the amount of I^{131} per mg. of fetal thyroid was 26 times the amount per mg. of fetal liver. On the

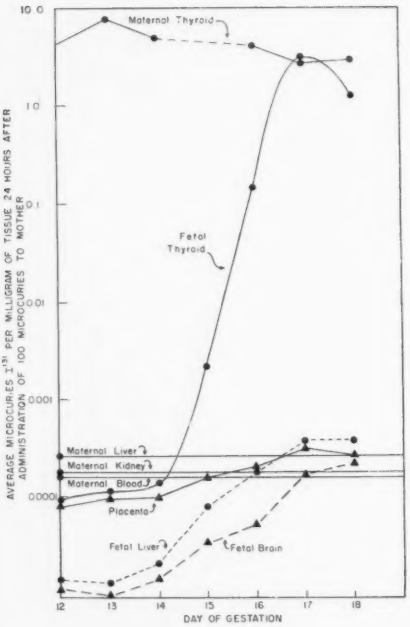

FIG. 1. The points are average figures for the fetuses and mothers analysed at each day of gestation. The numbers of mothers and fetuses considered are found in Table 1. No measurements are available for maternal thyroids on the 15th day of gestation.

16th day of gestation, the thyroids contained 767 times the liver concentration, and on the 17th day 7838 times as much. By the 18th day the amount concentrated by the fetal thyroid had fallen to 3178 times the liver concentration.

The amount of I^{131} per mg. of *maternal* thyroid generally decreased as gestation proceeded. At the 17th day the average concentration of iodine in the fetal thyroid slightly exceeded the average concentration in the maternal thyroid (Fig. 1, Table 1).

The content of I^{131} per mg. of fetal liver and brain increased from the 13th to 17 day of gestation and leveled off on the 18th day. The concentration of iodine in brain tissue was consistently less than in liver (Fig. 1). The μc. of radioiodine per mg. of placenta increased as gestation proceeded, reach-

ing a maximum on the 17th day. The iodine concentration was four times as great on the 17th day as on the 12th. On the 18th day the amount decreased by 20% of the 17th day value. The wet weight of the placenta varied in a similar fashion; increasing to the 17th day, then decreasing on the 18th day (from an average wet weight of 140 mg. on the 17th day to 117 mg. on the 18th day).

The amount of I^{131} contained per mg. of maternal liver, kidney, and blood varied only slightly from animal to animal. There was no correlation

TABLE 1. COMPARISON OF FETAL THYROID AND MATERNAL THYROID AVIDITY FOR IODINE ON A PER MILLIGRAM OF TISSUE BASIS

Day of gestation	(1) Number of pregnant mothers	(2) Maternal thyroid uptake %	(3) μc. of I^{131} per mg. maternal thyroid	(4) Number of fetuses	(5) Average μc. of I^{131} per mg. fetal thyroid	(5/3) Ratio of fetal thyroid uptake to maternal thyroid uptake
12	3	26.8	4.25	30	.00010	2.352×10^{-5}
13	5	48.5	7.70	50	.00012	1.558×10^{-5}
14	7	31.2	4.95	62	.00014	2.828×10^{-5}
15	6	—	—	51	.00221	—
16	4	25.7	4.08	40	.14420	3.534×10^{-2}
17	5	17.0	2.70	34	3.08821	1.144
18	4	18.3	2.90	31	1.25228	4.318×10^{-1}

between the iodine concentration in these tissues and the stage of gestation. The average concentrations are shown in Figure 1.

The percentage of the administered dose of radioiodine accumulated by *maternal* thyroids 24 hours following administration varied considerably between individuals at each day of gestation, but the trend was toward a lessened total uptake as gestation proceeded (Fig. 2). Toward term (16th, 17th and 18th days) some correlation between litter size and maternal thyroid iodine uptake could be noted.

When the percentage of administered iodine taken up at various days of gestation by the thyroids of the entire litter is compared with the percentage uptake by the maternal thyroid on these days (Fig. 3), it is clear that the maternal thyroid is in competition with the fetal thyroids for the available iodine. As the litter uptake increases near term, the maternal uptake decreases. Over this period the combined iodine uptake of the maternal and fetal thyroids remains fairly constant (Fig. 3).

Examination of serial sections of fetuses revealed that follicles do not appear in the thyroids at the 14th or 15th days of gestation (Figs. 5, 6, 7 and 8). At this time cords of cells are present that are later to give rise to follicles and there is no evidence of colloid formation. Follicles and colloid storage appear first at the 16th and 17th days of gestation. Thus, differentiation of the capacity to accumulate iodine precedes morphological differentiation of follicles and the appearance of colloid by more than a day in these mice.

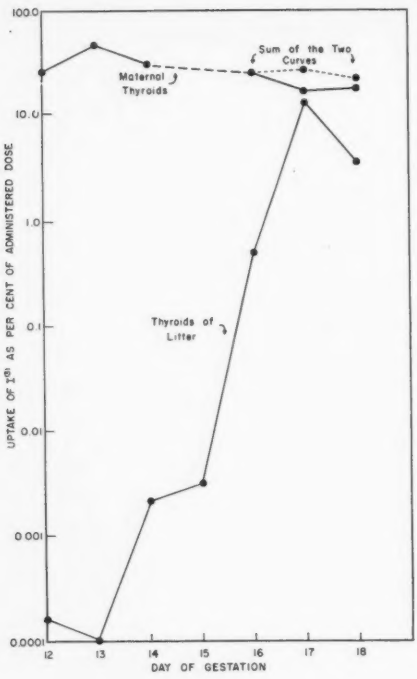

FIG. 2. The points are average figures at each day of gestation. Since the dose administered to the maternal organism was 100 μc. of NaI^{131}, the percentage uptake is equivalent to the total μc. accumulated.

The average wet weights of whole fetuses increased at a nearly constant rate to the 17th day of gestation. Between the 17th and 18th days the rate of increase in weight declined. A comparison of the increase in fetal thyroid weights on different days of gestation with the increase in fetal weights (Fig. 4) shows a divergence in growth rates at the 17th and 18th days. Table 2 compares the weights of fetuses, fetal thyroids and maternal thyroids at various days of gestation.

DISCUSSION

These experiments show, in units of concentration and in comparison to other fetal tissues, and to maternal tissues, the time in gestation when the fetal mouse thyroid commences to accumulate iodine, and give a picture of the gland's subsequent functional development. Of most significance are the comparisons that are possible among iodine uptakes of fetuses at differ-

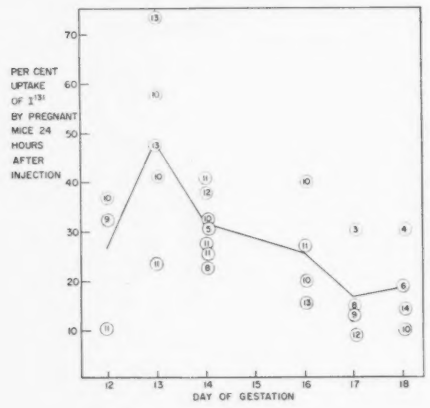

FIG. 3. Each circle represents the uptake of I^{131} by the thyroid of an individual pregnant mouse. The number within the circle is the number of fetuses that the mouse was carrying. The solid line is drawn through average values at each day of gestation. No measurements are available for the uptake by thyroids on the 15th day.

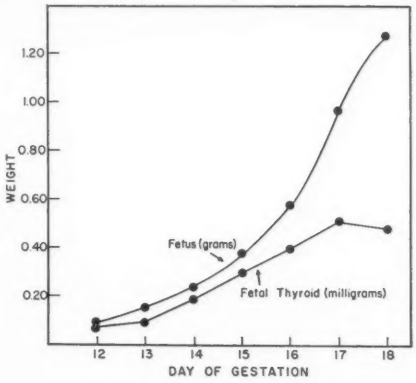

FIG. 4. The points are average values at each day of gestation. The numbers of fetuses weighed at each day can be found in Table 1.

ent stages of gestation, and among fetal and maternal tissues. Undue importance should not be attached to the absolute values for iodine uptake found in a study such as this. These figures are applicable to these specific experimental procedures. Radioiodine uptake varies with many factors, among them: the amount of stable iodine in the diet (3, 4, 5, and 6); time elapsed between administration of the iodine and assay of the fetuses; the environmental temperature (7, 8); and, of course, the amount of radioiodine administered to the mother. All of these factors were held constant in our experiments.

According to our findings the fetal mouse thyroid begins to concentrate iodine before the first appearance of follicles and colloid. This result is in accord with reports for other species. For example, iodine is concentrated in the thyroid before the appearance of follicles or colloid in the chick (9, 10), in the rabbit (11, 12), and in the calf (13).

The earliest time reported for concentration of iodine in the fetal human thyroid is the 12th week of gestation (14, 15). By the twelfth week, follicles are already beginning to form in the human thyroid. In the 7th week of gestation human thyroids are at least as differentiated as the mouse thyroid of the 15th day when iodine concentration has begun. Studies by Otis

TABLE 2. COMPARISON OF WEIGHTS OF FETUSES, FETAL THYROIDS, AND MATERNAL THYROIDS

Day of gestation	Fetal weight (mg.)	Fetal thyroid weight (mg.)	Maternal thyroid weight (mg.)	Ratio of fetal thyroid to maternal thyroid weight
12	97.4	.08	6.3	1.269×10^{-2}
13	154.5	.10	6.3	1.587×10^{-2}
14	237.8	.19	6.3	3.015×10^{-2}
15	379.2	.30	6.3	4.761×10^{-2}
16	582.7	.40	6.3	6.349×10^{-2}
17	975.8	.51	6.3	8.095×10^{-2}
18	1278.6	.48	6.3	7.619×10^{-2}

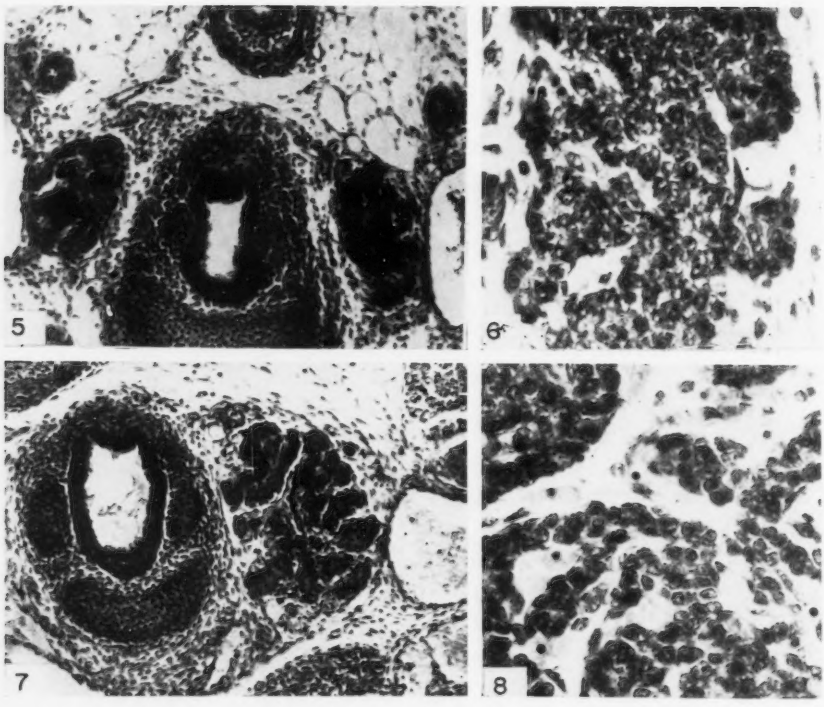

FIG. 5. Cross section through esophagus (at top), trachea (lower center) and thyroids (flanking trachea) of a mouse fetus at the 14th day of gestation. These structures are reproduced at the same magnification (×58) as the trachea and thyroid of the 15 day mouse fetus shown in Fig. 7. The size and vascularization can thus be directly compared.

FIG. 6. Mouse fetal thyroid tissue at the 14th day of gestation. ×140.

FIG. 7. Cross section through trachea (at left) and thyroid (at right) of a mouse fetus at the 15th day of gestation when the gland has begun to concentrate iodine. ×58.

FIG. 8. Mouse fetal thyroid tissue at the 15th day of gestation. Follicles and colloid are not yet present though the gland concentrates iodine at this time. ×140.

and Brent (16) correlating the time of appearance of a number of structures in the embryos of mice and human beings make it possible to compare equivalent stages of development in these two species. The time when the mouse first concentrates iodine in the thyroid, the 14th to 15th day of gestation, is comparable in human development to the 6th to 8th week of pregnancy. From these considerations one might suspect that the human fetal thyroid concentrates iodine earlier than the 12th week, as reported, and probably as early as the 6th to 7th week.

The total uptake of iodine by the fetal mouse thyroid is affected by the pattern of mass increase of the fetal thyroid as well as by the thyroid's changing capacity to concentrate iodine as gestation proceeds. Weight

measurements on successive days of gestation showed that the rate of growth of the fetal thyroid does not keep pace with the rate of increase of the fetus near term. Hall and Kaan (17) noted a similar situation in the rat.

Nearly every parameter that was measured showed an actual decrease, or a decline in the rate of increase, between the 17th and 18th days of gestation. Between these two days, the average weights of placentae and of fetal thyroids decreased; the concentration of iodine in the fetal thyroids and the placentae decreased; the average uptake of iodine by the thyroids of whole litters decreased; the rate of increase in the weights of the fetuses declined, as did the rate of increase in concentration of iodine in the fetal liver and brain. Since eight measured parameters exhibited changes of the same sign between the 17th and 18th days of gestation, and these changes were in each case contrary to the previous pattern of change, confidence that each individual change is significant is increased.

A logical probability is that placental changes between these two days are at least in part responsible for the other changes. The placental changes are most likely referable to the imminence of parturition. It was noted when dissecting out the fetuses that 18 day placentae are more readily detached from the uterus than 17 day placentae. The average weight of the placentae decreased between these two days, as did the iodine concentration in the placental tissue. The decrease in mass is very possibly accompanied by a change in diffusion area and dynamics of exchange between the mother and fetus, and this, together with the decrease in iodine concentration in the placenta, could lessen the availability to the fetal circulation of iodine from the maternal sources. This could account, at least in part, for the decrease in the concentration of iodine in the fetal thyroids between the 17th and 18th days. The decreased uptake of iodine by whole litters between these two days must be referable to both the decreasing iodine concentration and to the decreasing fetal thyroid weight. The possibility that there is a change in iodine turnover on the 18th day is less likely but is not ruled out. The placental changes most likely effect the exchange of many materials between the fetal and maternal organisms and could thus be responsible for the decline in the rate of growth of the fetus between the 17th and 18th days.

In guinea pigs, Postel (18) has demonstrated that very little radioiodine tagged triiodothyronine will pass the placenta into the fetal circulation when injected into the mother. In the same species, Lybeck and Hirvonen (19) found that little or no radiothyroxine will pass the placenta into the maternal circulation when injected into the fetus. Similarly in rabbits, Hall and Myant (20) demonstrated that the placenta is almost completely impermeable to labelled thyroxine up to the 19th day, but becomes more permeable thereafter. Such studies suggest that most administered radioiodide that passes the placenta must not do so bound up

in these compounds. Logothetopoulos and Scott (21), working with guinea pig, rabbit and rat, obtained data that suggest a placental transport mechanism for iodide. The mechanism is inhibited by thiocyanate. The I^{131} in fetal and maternal plasma was shown to be in the form of the free iodide ion.

Nataf *et al.* (22) reported concentration of radioiodine in the placenta following injection of I^{131} into pregnant rats. The accumulation of iodine in the placenta was greater when the iodine was injected into the mother than when it was injected into the fetus. They concluded that the placenta plays an important role in the regulation of the exchange of iodine between mother and fetus and this role is selective, favoring the transfer of iodine from mother to fetus at the end of gestation. These results and our own call attention to the possible modifying influences of placental factors in this kind of investigation of the functional development of the thyroid.

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THE BIOSYNTHESIS OF ADRENOCORTICAL HORMONES BY THE HUMAN ADRENAL GLAND *IN VITRO*[1]

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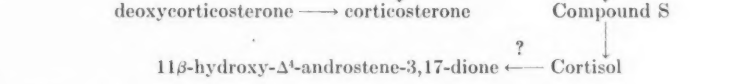
ABSTRACT

The results of a preliminary study on the biosynthesis of adrenocortical hormones by the human adrenal gland in vitro are presented. The substrates investigated were 3β-hydroxy-Δ^5-pregnene-20-one, progesterone, 17α-hydroxyprogesterone, compound S (17α,21-dihydroxy-Δ^4-pregnene-3,20-dione), and deoxycorticosterone. The results have led to the proposal of the following tentative scheme of adrenocortical biosynthesis by the human adrenal:



In 1949, Hechter *et al.* (1) first demonstrated the 11β-hydroxylation of deoxycorticosterone to form corticosterone by perfusion of the isolated bovine adrenal gland. Since then several biosynthetic reactions on steroid substrates have been demonstrated by the same group using the perfusion technique. These include the oxidation of the Δ^5-3β-hydroxyl group to Δ^4-3-ketone, 6β-hydroxylation, 11β-hydroxylation, 17α-hydroxylation, and 21-hydroxylation (2). These same reactions have been demonstrated in bovine adrenal enzyme preparations by Hayano and Dorfman, Plager and Samuels, Savard, Meyer, Haines, and others (2). More recently, Meyer (3) demonstrated the 19-hydroxylation of Δ^4-androstene-3,17-dione by the bovine adrenal.

The biosynthesis of adrenocortical hormones by the human adrenal has been actively investigated in our laboratories for the past several years. In a preliminary note (4), we reported the conversion of progesterone, 17α-hydroxy progesterone, and 11-deoxycortisol to cortisol by human adrenal tissue *in vitro*. These studies had demonstrated the 11β-hydroxylation, 17α-hydroxylation, and 21-hydroxylation of the steroid molecule by the human adrenal. A continuation of these studies with various steroid

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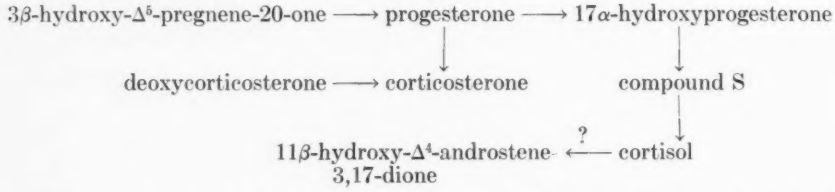
substrates have led to a scheme of adrenocortical synthesis by the human adrenal proposed below:



The experiments which led to the proposal of this scheme are described herein.

EXPERIMENTAL

Adrenal glands obtained from female patients with metastatic carcinoma of the breast were used for this study. In one case, an adrenal was obtained from a male with the same diagnosis. A total of twelve human adrenals were studied in this series. A section of each of the glands was removed for histologic examination. Slices were prepared from each of two of the glands while minces were prepared from each of the remaining glands. These preparations were placed in a medium containing 25–30 ml. of Krebs-Ringer-phosphate (5) solution (pH 7.4), 0.027 mmoles DPN, 0.02 mmoles ATP, and 0.68 mmoles sodium fumarate. Each gland, with the exception of two controls, was incubated with 5 mg. of steroid substrate added in approximately 0.3 ml. of ethyl alcohol. All preparations were incubated for three hours at 37° C with 95 per cent O_2-5 per cent CO_2 as the gas phase. The incubated samples were immediately set up for extraction with methylene chloride by a continuous dialysis and extraction technique previously described (6). The CH_2Cl_2 solutions were then evaporated to dryness in a water bath at 40° C in a fine stream of nitrogen. Each extract was distributed between 5 ml. of distilled water and 5 ml. of ethyl acetate in order to remove traces of salts. The water layer was extracted two additional times with 5 ml. of ethyl acetate. The ethyl acetate solutions were dried over anhydrous Na_2SO_4 and evaporated to dryness under vacuum. The dried extracts were subjected to paper partition chromatography in a combination of systems first described by Zaffaroni and Burton (7) and by Savard (8). The chromatographic sequence was so designed as to permit a complete separation of all possible intermediates from the substrate to the end products. The run-off from each chromatographic system was concentrated by evaporation in a water bath in a fine stream of nitrogen. This concentrate was then chromatographed in the next system. The chromatographic sequence, together with the solvent used to impregnate the paper and the standards employed for each system, is presented in Table 1. The paper employed was Whatman No. 1 washed by Soxhlet extraction for one week with water:methanol (3:1). All solvents were purified by distillation on fractionating columns. The techniques for the detection of steroid substrates on paper chromatograms, quantitative elution, and quantitative estimation by ultraviolet spectroscopy have been discussed elsewhere (6,9,10). The characteristic absorption spectrum of each steroidal substance in concentrated sulfuric acid from 220 to 600 mμ (11) was determined in every case. Solvent paper blanks corresponding to the exact area of that of the eluted substances were used without exception in quantitative estimation by ultraviolet spectroscopy and in the determination of the absorption spectrum in concentrated sulfuric acid. The quantitative estimation of steroids with an α,β-unsaturated-3-ketone was made on the basis of their optical densities at 240–241 mμ in absolute methyl alcohol using the extinction coefficient of the authentic steroid in the same solvent. All ultraviolet and visible spectrophotometric analyses were made upon a Beck-

TABLE 1. PAPER CHROMATOGRAPHIC SYSTEMS EMPLOYED TO SEPARATE STEROIDS

	Moving phase*	Stationary phase†	Development time (hours)	Standard
1.	Toluene	Propylene Glycol	96	Cortisol
2.	Toluene	Propylene Glycol	24–30	Cortisone
3.	Methyl Cyclohexane	N,N-dimethyl Formamide	72–96	Corticosterone, Reichstein's Compound S, 11β-hydroxy-Δ^4-androstene-3,17-dione
4.	n-Hexane	Propylene Glycol	24	11β-hydroxy-Δ^4-androstene-3,17-dione, 17α-hydroxyprogesterone, deoxycorticosterone
5.	n-Hexane	Propylene Glycol	10–12	Dehydroepiandrosterone, androsterone
6.	n-Hexane	Propylene Glycol	6	Δ^4-androstene-3,17-dione
7.	n-Hexane	Propylene Glycol	3	Progesterone

* The moving phase was always saturated with the stationary phase.
† The paper was impregnated with a 50% solution of the stationary phase in methanol.

man Model DK-2 automatic recording spectrophotometer. Melting point determinations were made on a Fisher-Johns melting point apparatus and are uncorrected. All infared analyses were made by the potassium bromide pressed disc technique upon a Perkin-Elmer Model 137 Infracord Spectrophotometer. The steroidal substance was transferred with acetone onto 15 mg. KBr, thoroughly ground and then vacuum dried. A micro pellet 4.5 mm. in diameter and 0.3 mm. thick was prepared from each sample with a vacuum die under a pressure of 20,000 lbs. The reference beam was always attenuated by a blank potassium bromide pellet comparable to that containing the steroidal substance.

RESULTS

In Table 2 are presented a summary of the results of these experiments. Experiments 1a and 1b are control experiments to which no substrate was added. The glands used in experiments 1a and 1b were normal as judged from histologic examination. Upon chromatography in system 1 (see Table 1) both controls yielded a spot which migrated like cortisol. It absorbed in the ultraviolet under the mineralight lamp and gave a posi-

TABLE 2. STEROIDS ISOLATED FROM HUMAN ADRENAL INCUBATION EXPERIMENTS

Experiment	Gland-weight (gm.)	Substrate (5 mg.)	Cortisol (μg.)	Corticosterone (μg.)	11β-hydroxy-Δ^4-androstene-3,17-dione (μg.)	17α-hydroxy-progesterone (μg.)	Progesterone (μg.)
1a	4.38	Control	370	79	147	0	0
1b	4.82	Control	312	43	106	0	0
2a	9.49*	3β-hydroxy-Δ^5-pregnene-20-one	1,312	243	0	0	0
2b	4.96	3β-hydroxy-Δ^5-pregnene-20-one	343	98	0	75	65
3a	6.52	Progesterone	801	387	64	437	—
3b	9.20†	Progesterone	118	182	0	311	—
4	9.29	17 α-hydroxy-progesterone	2,502	0	32	—	0
5	8.51‡	Compound S	871	0	0	0	0
6a	4.85	Desoxycorticosterone	54	1,757	0	0	0
6b	4.34§	Desoxycorticosterone	70	82	0	0	0
6c	7.13‖	Desoxycorticosterone	107	152	0	0	0

* Cortical adenoma.
† Hemorrhage and tumor involvement in adrenal.
‡ Carcinoma in adrenal, undifferentiated, secondary to carcinoma of female mammary gland.
§ Stored gland in frozen state for a period of 4 days.
‖ Stored gland in frozen state for a period of 15 days.

tive reaction to the blue tetrazolium reagent for an α-ketol. Both substances gave a single absorption maximum in absolute methyl alcohol at 241 mμ identical to that of authentic cortisol. When treated with concentrated sulfuric acid each substance produced a spectrum identical to that of cortisol with maxima at 240, 280, 390, and 475 mμ. The infrared spectrum of each substance showed an hydroxyl band at 3440 cm.$^{-1}$, a C-20 carbonyl band at 1710 cm.$^{-1}$, α, β-unsaturated carbonyl bands at 1650 cm.$^{-1}$ and 1615 cm.$^{-1}$, and bands identical to those of cortisol in the region 1400 cm.$^{-1}$ to 675 cm.$^{-1}$. The cortisol from both samples was extracted from the KBr pellets, pooled, and crystallized first from ethyl acetate-n-hexane and then from acetone-n-hexane. The substance melted at 203–206° and there was no depression of the melting point on admixture with authentic cortisol, melting at 206–207°.

In these control experiments, nothing was detected in chromatographic system 2 (Table 1). In chromatographic system 3, a substance with the mobility of corticosterone was detected under the mineralight lamp. It gave a positive test for an α-ketol with the blue tetrazolium reagent. The steroid-containing zones were eluted as described above. Both substances gave a single absorption maximum in absolute methanol at 240 mμ. In concentrated sulfuric acid both substances produced a spectrum identical to that of corticosterone with maxima at 285, 330, 373, and 455 mμ. These two substances were pooled and subjected to infrared analysis. The infrared spectrum showed an hydroxyl band at 3378 cm.$^{-1}$, a C-20 carbonyl band at 1694 cm.$^{-1}$, α,β-unsaturated carbonyl bands at 1645 cm.$^{-1}$ and 1613 cm.$^{-1}$, and bands identical to those of corticosterone in the region 1400 cm^{-1} to 675 cm.$^{-1}$.

A second substance was detected in both 1a and 1b in chromatographic system 3. It had the mobility of standard 11β-hydroxy-Δ^4-androstene-3,17-dione. It gave a negative reaction to the blue tetrazolium reagent for an α-ketol but it gave a typical positive reaction to the Zimmermann reagent for a 17-ketone. Both substances gave a single absorption maximum in absolute methanol at 240 mμ. In concentrated sulfuric acid, both substances produced a spectrum identical to that of 11β-hydroxy-Δ^4-androstene-3,17-dione with maxima at 285, 380, and 460 mμ and a shoulder at 400 mμ. These substances were pooled and subjected to infrared analysis. The infrared spectrum showed an hydroxyl band at 3350 cm.$^{-1}$, a 17 ketone at 1741 cm.$^{-1}$, α,β-unsaturated carbonyl bands at 1645 cm.$^{-1}$ and 1613 cm.$^{-1}$, and bands identical to those of 11β-hydroxy-Δ^4-androstene-3,17-dione in the region 1400 cm.$^{-1}$ to 675 cm.$^{-1}$.

No other steroidal substances were located in any of the other chromatographic systems in these control incubations.

In Experiments 2a and 2b, 3β-hydroxy-Δ^5-pregnene-20-one was used as substrate. The gland used in experiment 2b was a normal adrenal whereas the gland used in Experiment 2a had a cortical adenomata. The patholog-

ical diagnosis was carcinoma, undifferentiated in the right adrenal following carcinoma of the right male mammary gland. In both Experiments 2a and 2b, cortisol was isolated from chromatographic system 1 (Table 1). In both cases the identity of this substance as cortisol was established by the same criteria previously described for the control experiments, that is, spectrophotometric properties in absolute methyl alcohol and concentrated sulfuric acid, infrared analysis, and melting point determinations. It can be seen from Table 2 that in the case of the normal gland (2b), the amount of cortisol formed was not above the levels in the control glands without substrate. However, the gland with the adenoma (2a) showed an approximately 20% conversion of 3β-hydroxy-Δ^5-pregnene-20-one to cortisol. It is probable, therefore, that the adenoma was a highly functional tumor of the adrenal cortex since it was capable of transforming a substrate in a low state of oxidation to cortisol in good yield.

In chromatographic system 3 (Table 1), a substance with the mobility of corticosterone was detected in both 2a and 2b. These substances absorbed under the mineralight lamp and gave a positive test for α-ketols with the blue tetrazolium reagent. In both cases the substance was conclusively identified as corticosterone on the basis of chromatographic mobility, spectrophotometric properties in absolute methyl alcohol and concentrated sulfuric acid, and infrared analysis. It can be seen (Table 2) that in the case of the normal gland (2b), the amount of cortisosterone synthesized was only slightly above control levels. In the experiment with the gland containing the adenoma (2a), there was an approximately 4% conversion of the substrate to corticosterone. This again demonstrates the functionality of this type of adenoma.

In neither experiment 2a nor 2b was 11β-hydroxy-Δ^4-androstene-3,17-dione detected (Table 2). In experiment 2b (Table 2), however, a substance with the mobility of 17α-hydroxyprogesterone was detected in chromatographic system 4 (Table 1). This substance absorbed under the mineralight lamp and gave an atypical reaction to the Zimmermann reagent. It showed a single absorption maximum in absolute methyl alcohol at 240 mμ. On the basis of its optical density at this wavelength, 75 μg. were estimated to be present. In concentrated sulfuric acid, this substance showed maxima at 290 and 430 mμ identical to those for 17α-hydroxyprogesterone. Its infrared spectrum showed an hydroxyl band at 3380 cm.$^{-1}$, a C-20 carbonyl band at 1690 cm.$^{-1}$, α,β-unsaturated carbonyl bands at 1655 cm.$^{-1}$ and 1605 cm.$^{-1}$, and bands identical to those for 17α-hydroxyprogesterone in the region 1400 cm.$^{-1}$ to 675 cm.$^{-1}$.

In Experiment 2b also (Table 2), a substance with the mobility of progesterone was located in the chromatographic system 7 (see Table 1). The substance absorbed under the mineralight lamp and gave an atypical reaction to the Zimmermann reagent. After elution from the paper, the substance showed a single absorption maximum in absolute methyl alcohol

at 240 mμ on the basis of which 65 μg. were calculated to be present. In concentrated sulfuric acid, the substance showed a single absorption maximum at 290 mμ similar to that of authentic progesterone. The infrared spectrum was identical to that of progesterone.

In Experiments 3a and 3b (see Table 2) progesterone was used as the substrate. The adrenal used in Experiment 3a was normal while that used in Experiment 3b was hemorrhagic with tumor involvement. In both experiments cortisol was isolated from chromatographic system 1 (see Table 1). After elution, quantitative estimation, and pooling, the identity of this substance as cortisol was established by the same criteria previously described for the identification of cortisol in the control experiments. It can be seen from Table 2 that the normal gland (3a) converted progesterone to cortisol in fairly good yield (approximately 10%) whereas the gland with tumor involvement was non-functional.

In both experiments (3a and 3b) in which progesterone was used as substrate, corticosterone was isolated and identified on the basis of the criteria previously described. The normal gland (3a) synthesized 387 μg. of corticosterone (see Table 2) from 5 mg. of the substrate while the gland with tumor involvement synthesized 182 μg. of corticosterone.

A substance with the chromatographic mobility of 17α-hydroxyprogesterone was detected in chromatographic system 4 (see Table 1) in both Experiments 3a and 3b. In each case the substance absorbed under the mineralight lamp and gave an atypical reaction to the Zimmermann reagent on paper. After elution, the substance in each case showed a single absorption maximum in absolute methyl alcohol at 240 mμ. On the basis of their optical densities at this wavelength, 437 μg. were isolated from Experiment 3a and 311 μg. from Experiment 3b. In concentrated sulfuric acid, these substances showed maxima at 290 and 430 mμ identical to those of crystalline 17α-hydroxyprogesterone. The infrared spectra were identical to that of 17α-hydroxyprogesterone previously described. The remaining steroid from both experiments was pooled and crystallized from methanol. The substance melted at 211–214° and on admixture with authentic 17-α-hydroxyprogesterone, m.p. 213–216°, there was no depression of the melting point.

In Experiment 4 (see Table 2), 17α-hydroxyprogesterone was the substrate. In chromatographic system 1 (see Table 1), a substance with the chromatographic mobility of cortisol was detected. On the basis of its optical density at 241 mμ in absolute methyl alcohol, 2502 μg., were calculated to be present. The identification of this substance as cortisol was based on the same criteria previously described. This represents an approximately 50% conversion of the substrate to cortisol. With the exception of a small amount of 11β-hydroxy-Δ^4-androstene-3,17-dione (see Table 2), no other steroid except the substrate was isolated.

In Experiment 5, 17α,21-dihydroxy-Δ^4-pregnene-3,20-dione (Compound

S) was the substrate. Unfortunately, this adrenal was invaded by carcinoma, undifferentiated secondary to carcinoma of the female mammary gland. The only substance detected was cortisol. The amount isolated, 871 μg., represents an approximately 17% conversion of the substrate to cortisol. It was identified on the basis of the criteria previously described for the identification of this substance.

In Experiments 6a, 6b and 6c (see Table 2), 21-hydroxy-Δ^4-pregnene-3, 20-dione (deoxycorticosterone) was the substrate. Histologic examination of all three glands showed no abnormalities. However, the gland of Experiment 6a was a fresh gland, that of 6b was frozen for a period of 4 days while the gland used in 6c was frozen for a period of 15 days. It can be seen from Table 2 that only a very small amount of cortisol, much below control levels, was isolated from these experiments. In Experiment 6a, a substance with the chromatographic mobility of corticosterone was isolated from chromatographic system 3 (Table 1). On the basis of its optical density at 240 mμ, 1757 μg. were calculated to be present. This represents an approximately 35% conversion of the substrate to corticosterone. The identity of this substance as corticosterone was established on the basis of spectrophotometric properties in absolute methyl alcohol and concentrated sulfuric acid and infrared analysis as previously described. In addition, the substance was crystallized from ethyl acetate-n-hexane. It melted at 177–181° and there was no depression of the melting point on admixture with authentic corticosterone, m.p. 179–181°. There was very little conversion of the substrate to corticosterone in Experiments 6b and 6c (Table 2) indicating that freezing of the tissue had destroyed its ability to introduce an 11β-hydroxyl group into the substrate.

DISCUSSION

In every incubation experiment (see Table 2), with the exception of those where deoxycorticosterone was the substrate, cortisol was the major transformation product isolated and identified. The fact that cortisol is the major adrenocortical hormone elaborated by the human adrenal and isolated from human adrenal vein blood was been well established (10, 12, 13, 14). If we consider only the experiments in which the glands were shown to be histologically normal, it can be seen (Table 2) that progesterone (Experiment 3a) appears to be a better precursor than 3β-hydroxy-Δ^5-pregnene-20-one (Experiment 2b) in the biosynthesis of cortisol. In turn, 17α-hydroxyprogesterone (Experiment 4) is a better substrate than either progesterone or 3β-hydroxy-Δ^5-pregnene-20-one. Unfortunately, the gland used in Experiment 5 (Table 2) with Compound S was invaded by carcinoma. In spite of this, there was an approximately 17% conversion of the substrate to cortisol indicating that this substrate is a very efficient precursor of cortisol.

Corticosterone was isolated and identified from control experiments and

from experiments in which 3β-hydroxy-Δ^5-pregnene-20-one, progesterone, and deoxycorticosterone were the substrates. An examination of Table 2 indicates that deoxycorticosterone is the most efficient precursor of corticosterone. Although desoxycorticosterone has been isolated by Reichstein and von Euw (15) in 1938 from bovine adrenal tissue, it was not isolated as an intermediate in bovine adrenal perfusion experiments in which progesterone and 3β-hydroxy-Δ^5-pregnene-20-one were the substrates (16). The same is true of our incubation experiments with human adrenals. This could be explained by its rapid conversion into corticosterone as it was being formed from progesterone (16).

It is interesting to note that in Experiments 6b and 6c (Table 2), in which the glands were previously frozen for a period of 4 and 10 days respectively, only minute amounts of deoxycorticosterone were converted to corticosterone. This finding with human adrenals is in contrast to the findings of Hayano and Dorfman (17) that the loss of 11β-hydroxylating activity in stored beef adrenals could be regenerated by the addition of both ATP and DPN.

An examination of Table 2 also reveals that in an experiment with 3β-hydroxy-Δ^5-pregnene-20-one (2b) as substrate, both progesterone and 17α-hydroxyprogesterone were isolated as minor constituents. In both Experiments 3a and 3b with progesterone as substrate, 17α-hydroxyprogesterone was also isolated. In control Experiments 1a and 1b, 11β-hydroxy-Δ^4-androstene-3,17-dione was isolated and identified. This substance was also detected in one experiment with progesterone (Experiment 3a)

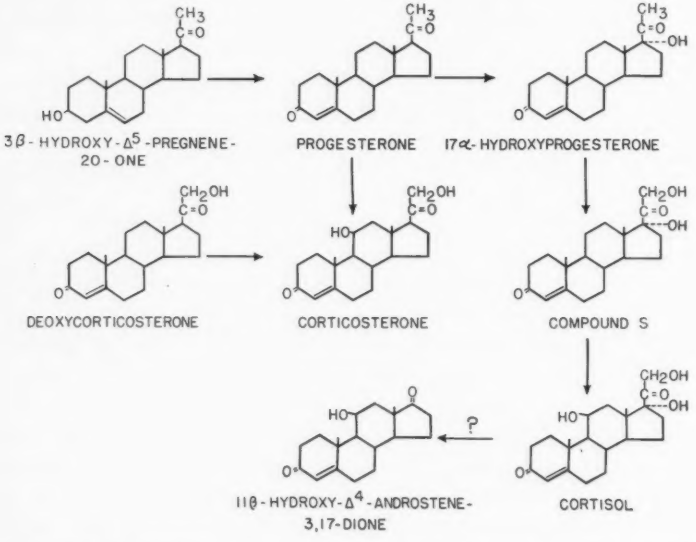


FIG. 1. Pathways in the biosynthesis of adrenocortical hormones by the human adrenal.

as substrate and in one experiment with 17α-hydroxyprogesterone (Experiment 4) as substrate. In both instances, however, the amount of 11β-hydroxy-Δ^4-androstene-3,17-dione found was much below that isolated from the control experiments. This would indicate that 11β-hydroxy-Δ^4-androstene-3,17-dione probably does not arise from any of the substrates studied in this series of experiments. Four explanations are plausible. It might arise through a biosynthetic route not involving C-21 adrenocortical hormones, it may be an artefact arising from the oxidative degradation of the side chain of cortisol, or the latter possibility may be mediated through an enzyme present in the adrenal. Finally, it might arise through the oxidative degradation of the side chain of 17α-hydroxyprogesterone followed by the 11β-hydroxylation of the Δ^4-androstene-3,17-dione thus formed.

On the basis of the above results, the most probable biosynthetic pathways to corticosterone and cortisol in the human adrenal are summarized in Figure 1. This scheme is very similar to that proposed by Levy *et al.* (16) on the basis of perfusion experiments with bovine adrenals.

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THE ISOLATION OF STEROIDAL SUBSTANCES FROM HUMAN ADRENAL VEIN BLOOD[1]

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ABSTRACT

A detailed analysis of 12 samples of human adrenal vein blood, collected from patients with mammary carcinoma, is reported. Cortisol has been isolated from every sample and identified. The isolation and identification of corticosterone in 6 instances, Compound S in 5 instances, and 11β-hydroxy-Δ^4-androstene-3,17-dione in 10 instances is also reported. The isolation of 17α-hydroxyprogesterone from 5 samples and dehydroepiandrosterone from 1 sample and their identification is reported for the first time.

BOTH *in vivo* and *in vitro* techniques have been utilized to study the metabolism of the steroid hormones in the last two decades. The *in vivo* studies have consisted mainly in the administration of large doses of tissue steroid hormones, especially to humans, followed by the isolation of the metabolic products from the urine. This technique has been supplemented by the administration of physiological concentrations of steroids labeled with isotopes. These methods have established an interrelationship between urinary steroid metabolites and those produced by a specific endocrine tissue. More recently, widespread use has been made of *in vitro* techniques comprising perfusion of the isolated intact glands and incubation experiments with tissue slices, tissue homogenates, and tissue enzyme preparations (1).

A more direct approach to the elucidation of the nature of the steroid hormones elaborated by a specific endocrine tissue is to study the venous effluent of that specific tissue. In 1950, Reich, Nelson, and Zaffaroni (2) reported finding cortisol and corticosterone in the adrenal vein blood of dogs stimulated with ACTH. This was followed with a report by Zaffaroni and Burton in 1953 (3) on the quantitative estimation of these two substances in the same medium. In addition, they reported the presence of several unidentified substances which gave a characteristic reaction for an α-ketol group at C-17. The presence of both cortisol and corticosterone in

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the adrenal vein blood of several species was established by Bush in 1953 (4).

The first report on the analysis of human adrenal vein blood was made by Sweat *et al.* (5) in 1953. By fluorometric methods he established the presence of 290 μg. of cortisol and 80 μg. of corticosterone per 100 ml. in the adrenal effluent of a patient with carcinoma of the breast and 850 μg. of cortisol and 236 μg. of corticosterone in a patient with Cushing's syndrome. That same year, Romanoff *et al.* (6) reported on the analysis of two samples of human adrenal vein blood, one consisting of 500 ml. from a male patient with metastatic carcinoma of the prostate, and a second consisting of 320 ml. from a female patient with metastatic carcinoma of the breast. Cortisol and corticosterone were isolated from the first sample in crystalline form and identified on the basis of chromatographic behavior, groups reactions, melting point and infrared data. In addition to cortisol and corticosterone, 11β-hydroxy-Δ^4-androstene-3,17-dione was identified in the second sample. Cortisone, Reichstein's Compound S (17α,21-dihydroxy-Δ^4-pregnene-3,20-dione), deoxycorticosterone, and progesterone were not found although diligently sought since the latter three compounds had been shown to be key intermediates in the biosynthesis of cortisol and corticosterone in *in vitro* studies with bovine adrenals (1). This was followed by a preliminary report from our laboratories in 1955 (7) on the isolation and quantitative estimation of cortisol and corticosterone in four samples of human adrenal vein blood.

The following is a continuation of these studies in which a detailed analysis of twelve samples of human adrenal vein blood is reported. The isolation from this medium and identification of Compound S, 17α-hydroxyprogesterone, and dehydroepiandrosterone is reported for the first time.

EXPERIMENTAL

Adrenal vein blood was collected from female patients with metastatic carcinoma of the breast. In one case, the blood was obtained from a male with the same diagnosis. Prior to adrenalectomy, the adrenal vein was cannulated and the effluent blood was collected. All blood samples were immediately extracted either by an alcohol-precipitation procedure (8), or by a simultaneous dialysis and extraction technique previously described (9). In either case, the methylene chloride solutions were washed four times with one-tenth the volume of cold 1N NaOH and five times with one-tenth the volume of cold deionized water to remove the phenolic fraction. The CH_2Cl_2 solutions containing the neutral fraction were evaporated to dryness in a water bath at 40° C in a fine stream of nitrogen. The dried extracts were subjected to paper partition chromatography in a combination of systems first described by Zaffaroni and Burton (10) and by Savard (11). The chromatographic systems employed and the sequence, together with the solvent used to impregnate the paper and the standards employed for each system, have been described (12). The techniques for the detection of steroid substances on paper chromatograms, quantitative elution, quantitative estimation by ultraviolet spectroscopy, and identification on the basis of sulfuric acid spectra and infrared analysis have also been discussed previously (12).

RESULTS

In Table 1 are presented a summary of the results of this study. In every case a substance with the chromatographic mobility of cortisol was detected in the 96-hour toluene-propylene glycol system (12). It gave a positive reaction to the blue tetrazolium reagent for an α-ketol and absorbed in the ultraviolet under the mineralight lamp. In every case, the substance possessed a single absorption maximum in absolute methyl alcohol at 241 mμ identical to that of authentic cortisol. Their concentrations were calculated on the basis of their optical densities at 241 mμ using the extinction coefficient of authentic cortisol in the same solvent. In concentrated sulfuric acid, the spectrum in each case was identical to that of

TABLE 1. STEROIDS ISOLATED FROM HUMAN ADRENAL VEIN BLOOD

Blood sample No.	Blood volume, ml.	Cortisol μg./100 ml.	Corticosterone, μg./100 ml.	Compound S μg./100 ml.	11β-hydroxy-Δ^4-androstene-3,17-dione, μg./100 ml.	17α-hydroxy-progesterone, μg./100 ml.	Dehydroepiandrosterone, μg./100 ml.
4L	258	56.2	24.0	9.8	53.9	0	0
6L*	262	143.9	8.5	21.0	132.3	0	0
7L	250	78.5	15.4	0	63.3	0	0
8L	245	35.3	0	0	25.3	0	0
9L	262	59.3	5.2	7.7	47.9	22.4	0
10L	63	119.7	0	10.0	0	0	0
11L†	258	28.1	0	5.0	0	5.4	0
12L‡	246	188.1	13.0	0	130.7	0	40.5
13L	120	162.3	22.2	0	17.7	21.0	0
14L	225	52.9	0	0	9.8	0	0
15L	220	5.6	0	0	6.1	20.4	0
16L§	205	13.7	0	0	135.0	16.3	0

* Histology showed cortical adenomata in adrenal.
† Hemorrhage and tumor involvement in adrenal.
‡ Psychotic patient.
§ Hyperplasia of adrenal cortex, nodular.

cortisol with maxima at 240, 280, 390, and 475 mμ. At this point, the cortisol fractions isolated from all the samples of adrenal vein blood were pooled and rechromatographed to insure homogeneity. Since only one spot was obtained corresponding to cortisol, it was subjected to infrared analysis by the potassium bromide pressed disc technique (12). The infrared spectrum was identical in every detail to that of cortisol previously reported (12). It can be seen from Table 1, that the levels of cortisol found in human adrenal vein blood are extremely variable.

In six out of the twelve adrenal vein blood samples studied (Table 1), corticosterone was detected in the chromatographic system methyl cyclohexane saturated with N,N-dimethylformamide. The substance gave a positive reaction to the blue tetrazolium reagent and absorbed in the ultraviolet under the mineralight lamp. In each case, the substance possessed a single absorption maximum in absolute methyl alcohol at 240

mμ and 4 points of maximum absorption in concentrated sulfuric acid at 285, 330, 373, and 455 mμ identical to those for authentic corticosterone. All the corticosterone fractions were pooled and rechromatographed to insure homogeneity. A single spot was obtained which on infrared analysis produced a spectrum identical to that of corticosterone previously described (12). It can be seen from Table 1 that the ratio of cortisol to corticosterone was extremely variable, ranging from approximately 2.3 (sample 4L) to 16.9 (sample 6L).

A substance with the chromatographic mobility of Compound S in the system methyl cyclohexane saturated with N,N-dimethyl formamide was detected in five of the twelve samples studied. In every instance the substance gave a positive reaction to the blue tetrazolium reagent for an α-ketol and absorbed in the ultraviolet under the mineralight lamp. In absolute methyl alcohol, the substance showed a single absorption maximum at 240 mμ and when treated with concentrated sulfuric acid produced a red coloration. In the latter solvent the substance produced points of maximum absorption at 240, 290, and 535 mμ and a shoulder at 340 mμ identical to authentic Compound S. All the Compound S fractions were pooled and rechromatographed. A single spot was obtained which on infrared analysis (12) showed an hydroxyl band at 3430 cm.$^{-1}$, a C-20 ketone at 1700 cm.$^{-1}$, α,β-unsaturated carbonyl bands at 1650 cm.$^{-1}$ and 1605 cm.$^{-1}$, and bands similar to those of Reichstein's Compound S in the region 1400 cm.$^{-1}$ to 675 cm.$^{-1}$. The ratio of cortisol to Compound S fell within a narrow range (5.6 to 7.7) in 4 of the 5 samples (4L, 6L, 9L, 11L) from which Compound S was isolated.

In ten instances (see Table 1), a substance with the chromatographic mobility of 11β-hydroxy-Δ^4-androstene-3,17-dione was detected in the system methyl cyclohexane saturated with N,N-dimethyl formamide (12). In every instance the substance absorbed in the ultraviolet under the mineralight lamp and gave a typical positive reaction to the Zimmermann reagent for a 17-ketone. In absolute methyl alcohol the substance gave a single absorption maximum at 240 mμ and in concentrated sulfuric acid produced a spectrum identical to that of 11β-hydroxy-Δ^4-androstene-3,17-dione with points of maximum absorption at 285, 380, and 460 mμ and a shoulder at 400 mμ. The ten fractions of 11β-hydroxy-Δ^4-androstene-3,17-dione were pooled, rechromatographed, and analyzed by infrared spectroscopy (12). The infrared spectrum obtained was identical to that of 11β-hydroxy-Δ^4-androstene-3,17-dione previously described (12). It can be seen from Table 1 that in six instances (4L-9L and 12L) the levels of 11β-hydroxy-Δ^4-androstene-3,17-dione were appreciable, the ratio of cortisol to 11β-hyroxy-Δ^4-androstene-3,17-dione ranging from approximately 1.0 to 1.4. In two cases (10L, 11L), none was found and in one instance (16L) the amount of 11β-hydroxy-Δ^4-androstene-3,17-dione found was approximately ten times as great as the cortisol level.

In five of the twelve blood samples, a substance with the chromatographic mobility of 17α-hydroxyprogesterone was detected in the system n-hexane saturated with propylene glycol (12). The substance gave an atypical Zimmermann reaction but it absorbed in the ultraviolet under the mineralight lamp. It had a single absorption maximum in absolute methyl alcohol at 240 mμ and showed two points of maximum absorption in concentrated sulfuric acid at 290 and 430 mμ identical to 17α-hydroxyprogesterone. The total material obtained from the five adrenal vein blood samples was pooled, rechromatographed and analyzed by infrared spectroscopy (12). The infrared spectrum was identical to that of 17α-hydroxyprogesterone previously reported (12). It can be seen from Table 1 that in two instances (15L, 16L) the levels of 17α-hydroxyprogesterone found were higher than the cortisol levels. It should be mentioned, however, that the levels of cortisol in 15L and 16L are the lowest ever found in our studies with human adrenal vein blood. The gland which produced blood sample 16L was not normal (Table 1) and unfortunately histology studies on the adrenal gland which produced blood sample 15L were not obtained.

In one of twelve human adrenal vein blood samples analyzed (12L), a substance with the chromatographic mobility of dehydroepiandrosterone was detected in the system n-hexane saturated with propylene glycol (12). It gave a typical reaction to the Zimmermann reagent for a 17-ketone and did not absorb in the ultraviolet under the mineralight lamp. In absolute methyl alcohol the substance did not show a point of maximum absorption but in concentrated sulfuric acid it showed points of maximum absorption at 305 and 410 mμ identical to crystalline dehydroepiandrosterone. The substance was subjected to infrared analysis by the potassium bromide pressed disc technique (12). It showed an hydroxyl band at 3335 cm.$^{-1}$, a 17-ketone band at 1740 cm.$^{-1}$ and bands identical to those for dehydroepiandrosterone in the region 1400 cm.$^{-1}$ to 675 cm.$^{-1}$. The amount of dehydroepiandrosterone found (Table 1) was estimated quantitatively by the Zimmermann reagent (13). This substance was isolated from human adrenal vein blood (12L) which came from a patient who, in addition to having mammary carcinoma, was a psychotic. An approximately comparable volume (218 ml.) of peripheral vein blood obtained from the same patient did not yield any dehydroepiandrosterone in the free neutral fraction.

DISCUSSION

It can be seen from Table 1 that cortisol is the only steroid hormone found in every sample of human adrenal vein blood studied. It has been isolated from the adrenal vein blood of several species (2–4). It is undoubtedly the main steroid substance elaborated by the human adrenal. This is in agreement with previous studies on human adrenal vein blood (5, 6, 7) and *in vitro* studies (12) in which cortisol was the major transformation

product isolated and identified. The levels of cortisol found were extremely variable. In this connection, one must consider that these were terminal cancer patients undergoing a variable degree of operative stress. In two instances (15L, 16L) the levels of cortisol were extremely low especially when one considers that levels as high as 1.0 mg. per 100 ml. have been found (7). However, the gland from which sample 16L was collected was not normal (Table 1) while histological studies were not obtained on the gland from which 15L was collected.

Corticosterone was isolated from six samples of adrenal vein blood while Compound S was isolated from five samples (Table 1). In three instances (4L, 6L, and 9L), corticosterone and Compound S occurred simultaneously. The levels of these two substances in human adrenal vein blood were somewhat comparable but in every instance much lower than the levels of cortisol. Corticosterone had been previously isolated from human adrenal vein blood (6, 7) and from that of other species (2–4). The presence of Compound S in 4 out of 5 samples of human adrenal vein blood had been indicated by Touchstone *et al.* (14) on the basis of chromatographic behavior of the free compound and its acetate and on the basis of sulfuric acid spectra. In this instance the isolation of Compound S has been confirmed by infrared spectroscopy.

11β-hydroxy-Δ^4-androstene-3,17-dione was isolated from ten out of the twelve samples of human adrenal vein blood. The levels were appreciable in six instances (4L–9L and 12L), the ratio of cortisol to 11β-hydroxy-Δ^4-androstene-3,17-dione ranging from approximately 1.0 to 1.4. In two instances none was found. In one instance (16L) the amount of 11β-hydroxy-Δ^4-androstene-3,17-dione was approximately ten times as great as that of cortisol, however, the adrenal, from which the latter sample of blood was collected, was not considered a normal gland (Table 1). This substance has also been isolated by Romanoff *et al.* (6) from human adrenal vein blood. *In vitro* studies with human adrenals (12), however, have demonstrated that it did not arise from C-21 substrates such as 3β-hydroxy-Δ^5-pregnene-20-one, progesterone, 17α-hydroxyprogesterone, or Compound S. Three possible explanations were considered (12). It might arise through a biosynthetic route not involving C-21 adrenocortical hormones, it may be an artefact arising from the oxidative degradation of the side chain of cortisol, or the oxidation of cortisol to 11β-hydroxy-Δ^4-androstene-3,17-dione may be mediated through an enzyme present in the adrenal and/or the blood.

It can be seen from Table 1 that 17α-hydroxyprogesterone was isolated from five samples of human adrenal vein blood. Its identity was conclusively established on the basis of its infrared spectrum. This is the first report of the isolation of this substance from adrenal vein blood. This is in excellent agreement with previous *in vitro* studies (12, 15) which had placed this substance in the position of an intermediate in the biosynthesis of cortisol by the adrenal. It can be seen from Table 1 that the levels of 17α-

hydroxyprogesterone were comparable to those of Compound S and corticosterone. It is interesting to note that it was also found in appreciable levels in the two samples of adrenal vein blood (15L and 16L) where the cortisol levels were very low.

It can be seen from Table 1 that dehydroepiandrosterone was isolated from only one sample of human adrenal vein blood. The identity of this substance as dehydroepiandrosterone was conclusively established on the basis of its infrared spectrum. This substance was not detected in the comparable free neutral fraction of a comparable volume of peripheral vein blood from the same patient. The isolation and identification of dehydroepiandrosterone from the hydrolyzed fraction of peripheral human plasma has been reported by Migeon and Plager (16) and by Clayton *et al.* (17). However, this is the first report of the isolation of this substance from the free neutral fraction of adrenal vein blood. It is indeed interesting that the patient, in addition to having carcinoma of the breast, was a psychotic.

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EFFECT OF OVINE INTERSTITIAL CELL-STIMULATING HORMONE ON SPERMATOGENESIS IN THE HYPOPHYSECTOMIZED MOUSE[1]

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ABSTRACT

The effect of interstitial cell-stimulating hormone (ICSH) on the maintenance of spermatogenesis in the C_3H mouse when administered immediately after hypophysectomy, and on the repair of the seminiferous tubules when administered 2 weeks after the operation, has been investigated. The hormone was injected for a period of 15 days; partial maintenance was obtained with a daily dose of 0.001 mg. and complete maintenance with 0.010 mg. With submaintenance doses, the higher the dose of the hormone the more mature were the germ cells observed in the tubules. When injections of ICSH were begun 2 weeks after hypophysectomy, repair of the germinal epithelium was obtained; degeneration and desquamation of the primary spermatocytes ceased, spermatogenic development in many tubules advanced to the spermatid stage, and the spermatozoa were observed in the epididymides in 50% of the injected animals. Interstitial cells, normal in appearance, were abundant. Testosterone propionate, 2.5 mg. per day, maintained spermatogenic activity; however, it effected only a slight repair of the tubules once the germinal epithelium had degenerated. Bovine growth hormone, itself ineffective in maintaining spermatogenesis, enhanced the effect of ICSH on the growth of the ventral prostate and seminal vesicles.

SPERMATOGENESIS is abolished in the male animal following hypophysectomy (1, 2); several gonadotropins as well as androgens can prevent degenerative changes in the seminiferous tubules if administered soon after the operation. The gonadotropins include human chorionic gonadotropin (HCG) (3), pregnant mare serum gonadotropin (PMSG) (4), and interstitial cell-stimulating hormone (ICSH) (5, 6); the role of follicle-stimulating hormone (FSH) in this process (7) remains to be estab-

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lished. Much of this information has come from studies conducted in the rat; however, Nelson and Merckel (8) have shown that several androgenic steroids can also maintain spermatogenesis in the mouse. The present investigation demonstrates the efficacy of ICSH as a stimulant to spermatogenic activity when administered to the hypophysectomized C_3H mouse either immediately following the operation or after a postoperative interval of 2 weeks.

EXPERIMENTAL PROCEDURE[5]

Male mice of the C_3H strain were hypophysectomized by the parapharyngeal approach at 42 days of age (9). In one series of animals, single daily injections were started on the day of operation and continued for a period of either 15 or 30 days; in a second series, they were started 2 weeks after hypophysectomy and continued for a period of 15 days. All injections were made subcutaneously. The protein hormones were dissolved in distilled water; the testosterone propionate was administered as a micro-suspension in a diluent that contained 0.4% Tween-80, 1% benzyl alcohol, and 1% NaCl. At autopsy, the ventral prostate, seminal vesicles and left testis were weighed on a Roller-Smith torsion balance. The right testis with its epididymis was fixed in Bouin's solution, subsequently embedded in celloidin, and sectioned at 6 micra. Central transverse sections of the testis stained with hematoxylin and eosin or with iron hematoxylin and aniline blue were selected for study.

The ICSH was an ovine preparation known as β-ICSH and shown to contain less than 0.1% of any biologically-active pituitary hormone (10). Three different preparations of bovine growth hormone were employed: the native hormone (11), the periodate-treated hormone (12), and the chymotrypsin-digested hormone (13). The testosterone propionate was purchased from the California Foundation for Biochemical Research.

RESULTS

Effect of Hypophysectomy. Before hypophysectomy, the adult mice showed a normal picture of spermatogenesis. At 42 days of age, approximately 20% of the seminiferous tubules in the testes contained mature spermatozoa and 70%–80% of the tubules were at the spermatid stage of development (Fig. 1A). The germ cells showed a similar distribution at 57 and at 72 days of age. Removal of the pituitary led to atrophy of the interstitial cells and marked degenerative changes in the tubules. Two weeks after hypophysectomy, the testicular and prostatic weights had fallen to 25% of their preoperative values (Table 1). In the seminiferous tubules, primary spermatocytes, many of them in various stages of degeneration, were the predominant germ cell type (Fig. 1B). In the epididymis of these animals (Fig. 2), there was a marked reduction in size of the lumina; the connective tissue was more prominent and had become dense and hyalinized. At the end of 4 weeks, the changes were more extensive. Interstitial cells could not be distinguished from fibroblasts, Sertoli cells were more in evidence than any germ cells, only a few tubules contained desquamating

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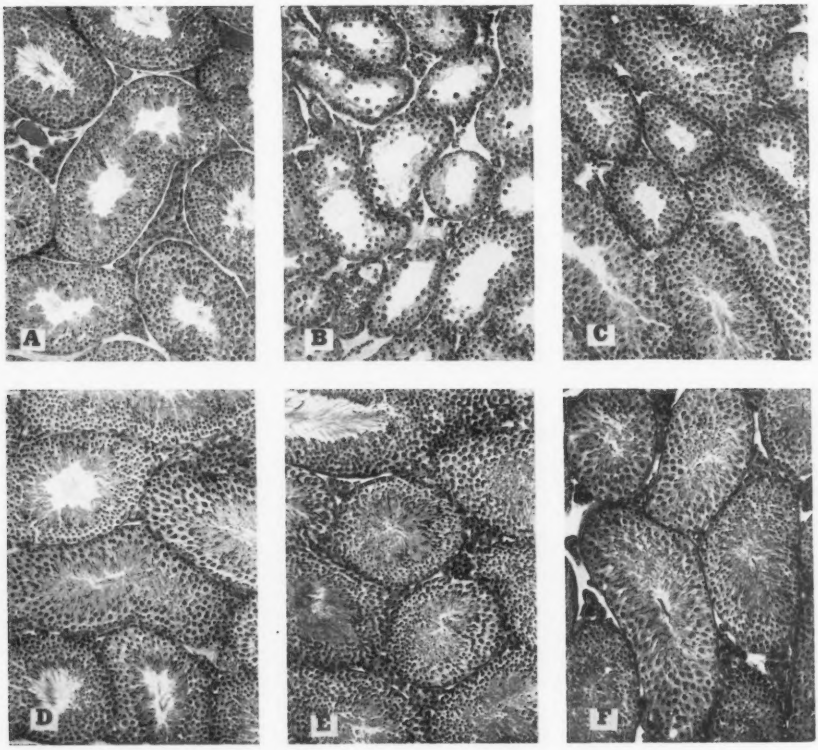

FIG. 1. Celloidin sections, 6 micra in thickness, of testes of C_3H mice hypophysectomized at 42 days of age and sacrificed at 57 days. Hormonal dosage represents dose level per day. Hematoxylin and eosin; ×80.

A. Normal control, 42 days of age. Spermatids and spermatozoa are present in all tubules. Interstitial cells are developed.

B. Hypophysectomized control. Spermatogonia and degenerating spermatocytes; interstitial cells are atrophic.

C. Injected with 0.001 mg. of ICSH. Secondary spermatocytes and spermatids in evidence.

D. Injected with 0.002 mg. of ICSH. Spermatids and immature spermatozoa predominate; interstitial cells mildly stimulated.

E. Injected with 0.100 mg. of ICSH. Maintenance of spermatogenesis equivalent to that in normal control; interstitial cells highly stimulated.

F. Injected with 0.100 mg. of ICSH and 0.100 mg. of growth hormone. Development similar to that obtained with 0.100 mg. of ICSH.

primary spermatocytes, and the diameters of the tubules were much reduced (Fig. 3A). Correspondingly, the weight of the testis decreased further from 17 mg. to 8 mg., whereas the weight of the ventral prostate remained comparable to that observed 2 weeks postoperatively.

Effect of Hormones on Maintenance. The effects obtained with several

dose levels of ICSH are illustrated in Figures 1C, 1D and 1E. Either 0.010 mg. or 0.100 mg. administered from the day of operation to sacrifice maintained the seminiferous tubules of the hypophysectomized animal in a state of development comparable to that of the normal control; the interstitial cells were abundant and epithelioid. Lower dose levels were not maximally effective. With 0.001 mg. and 0.002 mg., fewer spermatozoa were observed and a higher percentage of the tubules contained healthy but less mature germ cells; conversely, an increase in the number of degenerating cells was noted. At the same time, the differences observed with

TABLE 1. EFFECT OF MAINTENANCE TREATMENT WITH TESTOSTERONE PROPIONATE, ICSH AND GROWTH HORMONE ON THE WEIGHTS OF THE REPRODUCTIVE ORGANS OF HYPOPHYSECTOMIZED MALE MICE*

Treatment	Daily dose	No. of mice	Body weights		Organ weights			Maximal development of germ cells in testis
			Onset	Autopsy	Ventral prostate	Seminal vesicles	Left testis	
	mg.		gm.	gm.	mg.	mg.	mg.	
Group A								
Onset Control	0	10	21	21	6.5±0.4†	101±5	70±3	Spermatozoa
Terminal Control	0	8	18.5	22	7.9±0.7	126±6	70±2	Spermatozoa
Operated Control	0	20	19.5	16	1.3±0.1	11±1	17±1	Primary Spermatocytes
Testosterone Propionate	2.5	10	19.5	17	8.1±0.8	168±9	55±2	Spermatozoa
Growth Hormone	0.1	15	19.5	19.5	2.2±0.1	19±1	52±2	Spermatozoa
Growth Hormone (Periodate-Treated)	0.1	6	20	19	1.4±0.1	12±1	20±1	Primary Spermatocytes
Growth Hormone (Chymotryptic-digest)	0.1	6	19	17	1.6±0.1	11±1	18±1	Primary Spermatocytes
ICSH	0.2	6	24	22	2.3±0.2	16±1	22±1	Primary Spermatocytes
ICSH	0.001	6	19	16	1.7±0.1	11±1	37±3	Immature Spermatozoa
ICSH	0.002	6	19	16	1.8±0.1	15±1	55±3	Spermatozoa
ICSH	0.010	6	19	16	3.3±0.3	32±7	58±1	Spermatozoa
ICSH	0.100	6	21	19	5.6±0.2	78±6	69±3	Spermatozoa
Group B								
Terminal Control	0	10	21.5	25.5	11.4±0.8	162±9	83±4	Spermatozoa
Operated Control	0	11	21	17.5	1.4±0.2	12±1	8±1	Spermatogonia
Growth Hormone	0.1	4	21	22.5	1.6±0.3	11±1	25±3	Spermatids
ICSH	0.1	5	21	19	3.8±0.4	50±9	56±5	Spermatozoa
Growth Hormone + ICSH	0.1 + 0.1	5	22	24.5	9.2±0.4	115±8	56±2	Spermatozoa

* Mice hypophysectomized at 42 days of age; single subcutaneous injections begun day of operation. Group A, injections continued for 15 days; Group B, injections continued for 30 days.
† Mean ± standard error.

0.001 mg. and 0.002 mg. were striking (Table 1). In the first instance, the testicular weight was twice that of the hypophysectomized control and a majority of the tubules contained spermatids; in the second instance, the testicular weight was tripled and many immature spermatozoa were present along with a few mature forms.

As indicated in Table 1, testosterone propionate and growth hormone at dose levels of 2.5 mg. and 0.100 mg., respectively, likewise maintained the testicular weight as well as spermatogenic activity. The results obtained with testosterone propionate were anticipated; those obtained with native growth hormone suggested that a minute contamination with ICSH might be responsible for the observed effects. Indeed, such appears to be the case, for periodate-treated and chymotrypsin-treated preparations of growth hormone were ineffective in ameliorating the degenerative changes in the testis that ensue following hypophysectomy, although they retained their

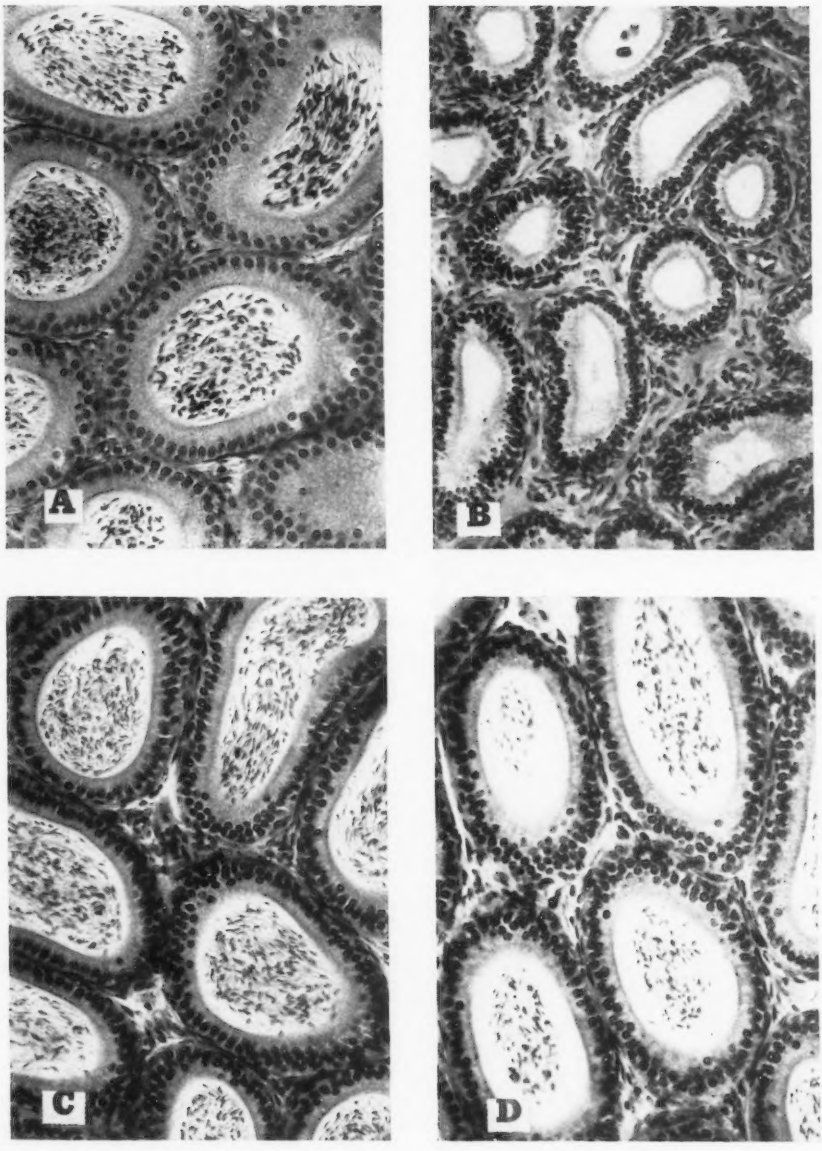

FIG. 2. Celloidin sections, 6 micra in thickness, of epididymis of C_3H mice hypophysectomized at 42 days of age and sacrificed at 72 days. Hormonal dosage represents dose level per day. Hematoxylin and eosin; ×225.

A. Normal control.

B. Hypophysectomized control. Note predominance of connective tissue and empty lumina.

C. Injected with 0.100 mg. of ICSH. Epididymal cells are hypotrophic and have crowded, dark nuclei; lumina contain spermatozoa.

D. Injected with 0.100 mg. of ICSH plus 0.100 mg. of growth hormone. Development comparable to that of normal control; in the nuclei of epididymal cells the chromatin is finely distributed.

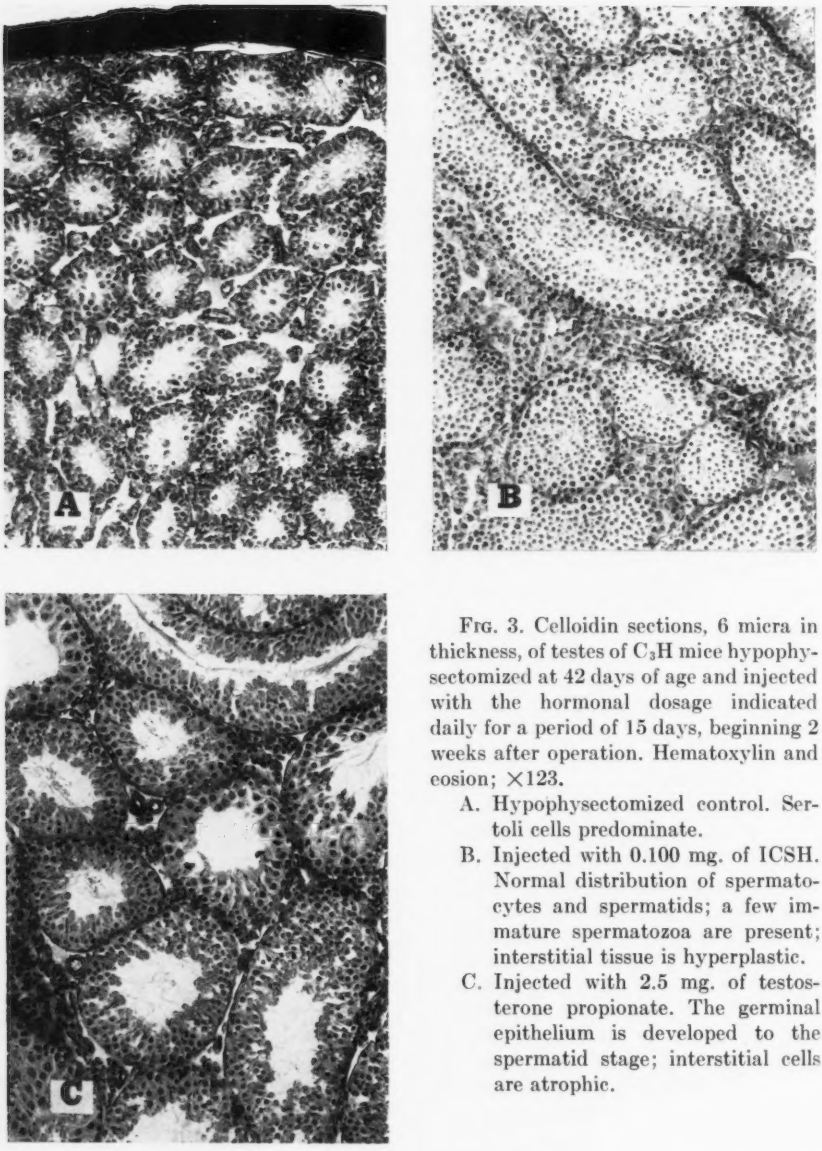

FIG. 3. Celloidin sections, 6 micra in thickness, of testes of C_3H mice hypophysectomized at 42 days of age and injected with the hormonal dosage indicated daily for a period of 15 days, beginning 2 weeks after operation. Hematoxylin and eosion; ×123.

A. Hypophysectomized control. Sertoli cells predominate.

B. Injected with 0.100 mg. of ICSH. Normal distribution of spermatocytes and spermatids; a few immature spermatozoa are present; interstitial tissue is hyperplastic.

C. Injected with 2.5 mg. of testosterone propionate. The germinal epithelium is developed to the spermatid stage; interstitial cells are atrophic.

capacity to stimulate body and skeletal growth. It was in combination with ICSH that growth hormone exerted its characteristic effect; a synergistic action of the two hormones in stimulating growth of the ventral prostate and seminal vesicles is illustrated in Table 1, where it can be seen that the administration of either 0.100 mg. of ICSH plus 0.100 mg. of growth hor-

mone, or 2.5 mg. of testosterone propionate, was the only hormonal treatment that stimulated growth of the ventral prostate and seminal vesicles parallel to that observed in the normal animal.

Effect of Hormones on Repair. By 15 days after hypophysectomy, the ventral prostate and seminal vesicles were atrophic and the seminiferous tubules of the testis contained no spermatozoa, spermatids, or secondary spermatocytes; only primary spermatocytes, many of them desquamating, were in evidence. The administration of 0.100 mg. of ICSH reversed the spermatogenic pattern from one of progressive degeneration to one of active reconstitution. Nearly every tubule showed a normal distribution of healthy immature forms (Fig. 3B); spermatogenic development in many tubules had advanced to the spermatid stage and, in one half the group,

TABLE 2. EFFECT OF DELAYED TREATMENT WITH TESTOSTERONE PROPIONATE AND ICSH ON THE WEIGHTS OF THE REPRODUCTIVE ORGANS OF HYPOPHYSECTOMIZED MALE MICE*

Treatment	Daily dose	No. of mice	Body weights		Organ weights			Maximal development of germ cells in testis
			Day of Operation	At autopsy	Ventral prostate	Seminal vesicles	Left testis	
	mg.	gm.	gm.	gm.	mg.	mg.	mg.	
Normal Control	0	10	21.5	25.5	11.4±0.8†	162.0±9	83.0±4	Spermatozoa
Hypophysectomized Control	0	11	21	17.5	1.4±0.2	11.8±0.4	7.9±0.5	Spermatogonia
Testosterone propionate	2.5	7	22	19	6.8±0.6	112.0±1.6	22.6±0.9	Spermatozoa
ICSH	.1	12	21	21	4.7±0.3	65.6±1.3	31.8±2.7	Spermatozoa

* Mice hypophysectomized at 42 days of age; single daily subcutaneous injections begun on day 57 and continued until sacrifice at 72 days.
† Mean ± standard error.

spermatozoa were observed in the epididymis. Interstitial cells were abundant and appeared normal. As illustrated in Figure 3C, 2.5 mg. of testosterone propionate also arrested the tubular degeneration; however, the development of the germ cells was far from normal and quite sporadic. Many tubules contained scattered cell types, i.e., primary spermatocytes along with immature spermatozoa, and very few showed the progressive cellular development seen in normal animals and in the hypophysectomized mice given ICSH. Many immature germ cells were also observed in the lumina of the epididymis, further indicating that the spermatogenesis was abnormal.

DISCUSSION

Androgens are known to maintain spermatogenesis for substantial periods of time in the absence of pituitary hormones. The original observations of Walsh, *et al.* (14), were soon confirmed with crystalline hormones (15), and subsequent studies have demonstrated that the action of the androgen is a direct one on the tubules (16). Gonadotropins which stimulate the interstitial cells of the testis to secrete androgen exert similar effects in hypophysectomized animals (4, 5, 6). In the present study, a daily dose of 0.010-0.100 mg. of purified ICSH maintained spermatogenesis in the mouse; similar results have been reported for the rat (6). Furthermore,

ICSH perpetuated spermatogenic activity in the mouse throughout the longest interval of time studied, namely 30 days. Neither ICSH nor androgen had an all-or-none effect on the development of the tubules; rather, with sub-maintenance doses of the hormones the tubules contained germ cells at varying degrees of maturation. The higher the dose of the hormone, the more mature were the germ cells. For example, with 0.001 mg. of ICSH secondary spermatocytes and spermatids were most in evidence; with 0.002 mg., spermatids and immature spermatozoa were most frequently observed. In the latter instance, a few mature spermatozoa were also present. Although it is tempting to deduce from these and earlier observations that the output of androgen from the interstitial cells does serve to regulate the intensity of spermatogenic activity in the seminiferous tubules as well as the rate at which it progresses, it remains to be demonstrated that such a relationship does in fact exist.

Earlier studies with crystalline hormones failed to demonstrate that androgens can reinstate the formation of spermatozoa after degenerative changes have occurred in the testicular tubules (15, 17). However, subsequently Nelson (18) has shown that testosterone propionate at a daily dose level of 2–3 mg. will cause the resumption of sperm formation when administered to rats 22–28 days after hypophysectomy. Smith and Leonard (3) obtained similar results with the gonadotropin from pregnancy urine, Greep and Fevold (5) with ICSH, and Nelson (15) with PMSG. On the other hand, Simpson, *et al.* (19), observed no effect on the germinal epithelium of the tubules with daily doses of ICSH as high as 0.50 mg. The latter results are in contrast to those reported here where the administration of 0.100 mg. of ICSH for a period of 15 days reinstated spermatogenic activity in the seminiferous tubules of the hypophysectomized mice, reversing the degenerative pattern attributable to hypophysectomy.

Although ICSH can effect repair of the seminiferous tubules, it cannot maintain the weights of the sex accessory glands over an extended period of time. A similar situation pertains in the Sprague-Dawley rat (20). In that strain of rat, as in the C_3H mouse, growth hormone acts synergistically with ICSH to stimulate growth of the ventral prostate and testes (21). Likewise, growth hormone acts synergistically with adrenocorticotropic hormone (ACTH) to promote growth of the adrenal gland in the rat (22) and in the mouse (23). Although the mechanism is not understood, it is clear that growth hormone acts as a biological synergist (24) in many physiological processes.

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THE SIGNIFICANCE OF THE BINDING OF INSULIN-I^{131} TO CYTOSTRUCTURAL ELEMENTS OF RAT LIVER

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ABSTRACT

Insulin-I^{131} has been intravenously administered in tracer doses to rats and its intracellular distribution determined. It was found that the major portion of the intracellular I^{131} was bound to mitochondria and microsomes. This bound insulin-I^{131} was completely precipitable with TCA and could not be removed by washing. Furthermore, in comparison to unbound insulin-I^{131}, it was less susceptible to endogenous degradative processes as well as those accomplished with added "insulinase." The significance of these observations on the hepatic fate of insulin-I^{131} is discussed in reference to the insulin economy of the body, as a whole, and the liver, in particular.

IN MAN the endocrine pancreas secretes its hormones directly into the portal vein as well as into the splenic vein. These secretions must then pass through the liver before being distributed systemically. In lower animals the same, or similar, anatomical pattern prevails. The significance of these facts is that, with the exception of the exocrine pancreas, the liver is the organ through which the total insulin secretion must pass before exerting its effects elsewhere. Consequently, the role of the liver in the fate of endogenously secreted insulin may be determinative; the importance of this has been most recently shown by Madison and Unger who demonstrated that the slow infusion of insulin into the portal vein would cause a decrease in hepatic glucose output without affecting muscle uptake (1).

Using I^{131} labeled insulin, a number of studies have been executed which serve to illuminate the hepatic fate of the hormone. *In vitro* perfusion experiments have shown that 22% of the insulin-I^{131} added to the perfusate is removed in a single cycle (2); similarly, in man intraportal injection at surgery followed by brachial artery sampling resulted in a value of 54% (3). These results were to be expected in view of earlier demonstrations of the concentration of insulin-I^{131} by the liver in man as well as lower animals (4). Underlying this rapid removal from the blood is the firm binding of insulin-I^{131} to the liver, and particularly its intracellular penetration and binding to cytoplasmic structures (5). Furthermore, the liver rapidly degrades the labeled hormone by a semi-specific enzyme system (6). In this

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regard liver and kidney are responsible for the very much larger proportion of insulin-I^{131} degraded *in vivo* (7) although muscle may also have a quantitatively significant role (8).

In view of these observations it would be of some interest to inquire into their significance relative to the insulin economy of the organism. The goal of the investigations reported in this paper has been a limited aspect of this interest, namely, to examine the relationship between insulin-I^{131} binding to cytoplasmic structures of rat liver and insulin-I^{131} degradation.

METHODS

Male albino rats of the Sprague-Dawley strain, weighing from 200–260 gm., were lightly anesthetized with ether and the insulin-I^{131} injected into one of the tail veins. The dose ranged from 0.11–0.28 insulin units/kg. and was equivalent to 2.8–4.4 μc. Five minutes later the livers were thoroughly perfused (5) and homogenized in 3 volumes of cold 0.25*M* sucrose. The homogenates were then diluted with sucrose solution to a final concentration of 5% and subjected to a differential centrifugation scheme (Fig. 1) similar to that of deDuve and coworkers (9). Nitrogen, deoxyribo- and ribonucleic acids, succinoxidase and glucose-6-phosphatase analyses were in agreement with values reported by these authors, thus indicating a reasonably clear separation of nuclear (N_w), mitochondrial (M_w), and microsomal (Mc) elements from each other and from the soluble cytoplasmic residuum (R).

In order to evaluate the role of the non-physiologic metabolism of insulin-I^{131} in the liver and its passive adsorption and release from cytoplasmic particulates, a second and simultaneously executed series of experiments was conducted. These were identical in all regards to the procedures and details outlined above except that the insulin-I^{131} was added to the homogenate, or to its various subfractions, in place of intravenous injec-

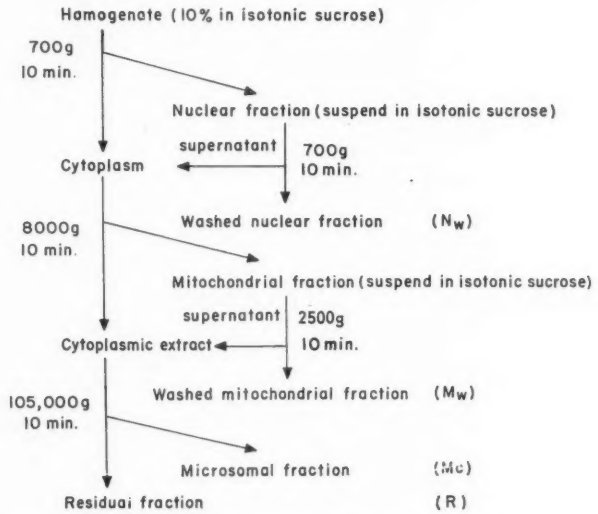

FIG. 1. Flow sheet for the centrifugal separation of cytostructural elements of rat liver.

tion into the animal. This second group of studies has been designated as the *in vitro* series in the remainder of this report and the first as the *in vivo* series.

The degradation of insulin-I^{131}, bound *in vivo* to cytoplasmic particulates, into fragments soluble in trichloracetic acid was investigated by incubating labeled mitochondrial and microsomal suspensions in the presence and absence of a purified rat liver "insulinase" preparation (10). The suspensions were prepared in phosphate-buffered (0.1*M*, pH 7.4) isotonic sucrose solution and 2 ml. aliquots, equivalent to the particulates from 100 mg. of liver, were added to 20 ml. beakers. After addition of 0.1 ml. of enzyme, also equivalent to 100 mg. of liver, or of 0.1 ml. of buffer, incubation was carried out in a Dubnoff bath at 37° in an air atmosphere. The reaction was stopped by the addition of an equal volume of 10% trichloroacetic acid (TCA) and the TCA-soluble I^{131} determined. Identical studies with unlabeled particulates were carried out by adding insulin-I^{131} in amounts equivalent to those found for the *in vivo* labeled particles, to the suspensions immediately prior to incubation.

Radioassay procedures were conventional, being done with a well-type scintillation counter and employing the usual corrections as required. Insulin-I^{131} degradation was evaluated by determining for each preparation that fraction of the total I^{131} remaining in solution in 5% TCA, the TCA-soluble fraction representing degradation products and the TCA-precipitable fraction the intact radio-iodinated molecule (4, 11).

RESULTS

The intracellular distribution of insulin-I^{131} in rat liver. In Table 1 are presented the intracellular distribution values for both the *in vivo* and *in vitro* series. The washed nuclear fraction (N_w) has little cytologic meaning since it is significantly contaminated with intact cells, erythrocytes and some cytoplasmic elements. However, the washed mitochondrial (M_w) and the microsomal (M_c) fractions are well defined and some degree of cytologic significance may be associated with the properties measured. To these two fractions a total of 66% of the intracellular I^{131} was bound in the *in vivo* series in comparison to 18% for the *in vitro* series. Furthermore, approximately 50% of the intracellular I^{131} was bound to a single cytologic entity, the microsomal fraction. In this regard it is important to point out that all of such bound radioactivity was precipitable in 5% TCA.

In order to establish more rigorously the firmness of the bond holding the labeled moiety to the cytostructures, particulates labeled *in vivo* and *in vitro* were studied with washing experiments by radioassaying mitochondrial and microsomal preparations after each of 3 additional sedimentations in 0.25*M* sucrose. Two procedural details were used to minimize the passive readsorption of any radioactivity released from the particulates: 1) all preparations were rehomogenized after each sedimentation in 25–100 vol-

TABLE 1. THE DISTRIBUTION OF RADIOACTIVITY IN RAT LIVER FOLLOWING INSULIN-I^{131} INJECTION

	n	% dose per gm.	Distribution, %				Recovery, %
			N_w	M_w	M_c	R	
In vivo series	32	3.16±0.42*	7.0±1.6	17.3±3.3	48.9±4.5	26.8±3.7	99.8±4
In vitro series	6	—	7.4±0.7	2.7±0.2	15.4±1.3	73.2±2.6	98.2±1.

* Mean ± standard deviation.

umes of ice-cold isotonic sucrose and 2) a comparison *in vivo* series was carried out in which a 2-hour incubation at 4° C was inserted prior to the beginning of the additional sedimentations.

The results of a typical experiment are presented in Figure 2. These data are interpreted as strongly supporting the inference that the hepatic metabolism of insulin-I^{131} involves its firm binding to intracellular structures. For example, when binding to microsomes could be attributed exclusively to passive adsorptive phenomena (*in vitro* series) more than 80% of the radioactivity was removed by 3 additional sedimentations whereas binding

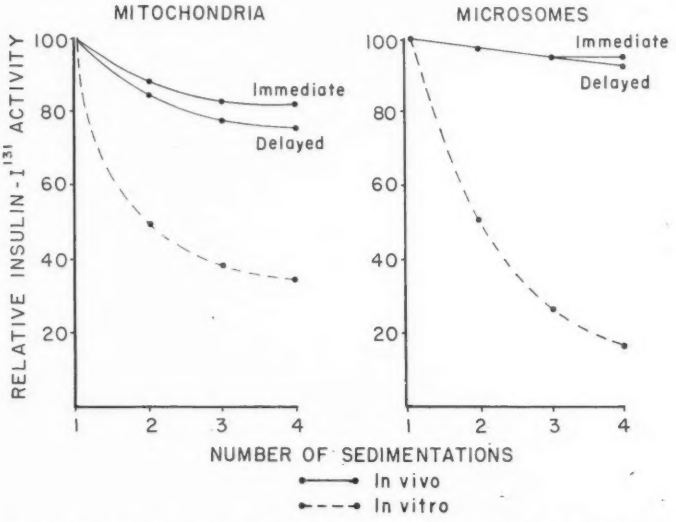

FIG. 2. The effect of washing on the retention of insulin-I^{131} by cytostructures of rat liver. Each point is the average of duplicate determinations.

under conditions of intact cellular physiology (*in vivo* series) permitted a contrasting loss of only 6%; this difference was further emphasized by the fact that incubation of the microsomes in a large volume of suspending medium had no significant effect on the I^{131} loss. Substantially the same observations were made for the mitochondrial fraction, the quantitative values being less striking but the same in kind as for the microsomes. Similar studies with both fractions were carried out in which the role of suspending medium was tested by substituting 0.85% saline or 0.88 *M* sucrose for 0.25 *M* sucrose and similar results were obtained.

The fate of insulin-I^{131} bound to cytostructural elements of rat liver. A clue to the intracellular fate of insulin-I^{131} in rat liver was obtained from the distribution studies cited above. It was found that the TCA-soluble I^{131} in the residual fraction increased several fold during the particle isolation

from the *in vitro* series whereas the same determination made for the *in vivo* series showed no increase. These observations were validated by the serial sampling of both types of preparations, *in vivo* and *in vitro*, over a 1 hour period and determining the amount of insulin-I^{131} degraded to TCA-soluble fragments; these operations were carried out at 0° C as well as 37° in order to establish the possible effect on the distribution values of such degradative activities during the particle isolation procedure. Data for a typical experiment are presented in Figure 3. It is apparent that at 0° C there was no significant increment in TCA-soluble I^{131} over a 60-minute incubation for the *in vivo* series, the actual values being 14.7% at zero time and 16.8% at 60 minutes. For the *in vitro* series, however, the increment was distinctly significant, the corresponding values being 14.7% and 27.1%. Essentially the same relationships obtained for the incubations at

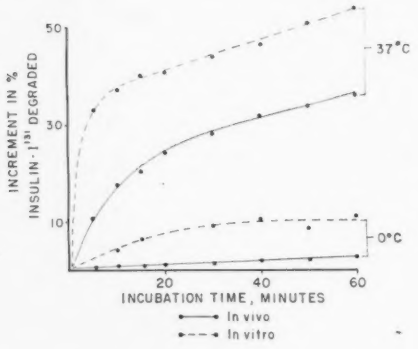

FIG. 3. The degradation of insulin-I^{131} in whole homogenates of rat liver. Each point is the average of duplicate determinations of a single homogenate preparation.

37°, the *in vivo* series showing a much smaller increment than the *in vitro* series. With reference to the data presented in Table 1, these observations led to the inference that insulin-I^{131} bound to cytostructural elements was relatively unavailable to the degradative activities of the cell.

Two broad mechanisms have been implicated in the degradation of insulin-I^{131} by liver; 1) a non-proteolytic process, presumably effective by means of the reductive action of —SH compounds formed *in vivo* and *in vitro* (12) and 2) a proteolytic process mediated by a semi-specific enzyme, "insulinase" (6). In order to test the hypothesis stated above it was necessary to evaluate the role of both degradative mechanisms. This was accomplished by incubating washed mitochondria and microsomes from the *in vivo* series at 37° with and without added "insulinase" and determining the amount of insulin-I^{131} rendered TCA-soluble; such determinations were contrasted with similar incubations of unlabeled particles to which insulin-I^{131} was added *in vitro*. It is important to note at this point that no "insulinase" activity is associated with the particulate elements sedimentable by the scheme employed in this study (13). Hence, the formation of TCA-

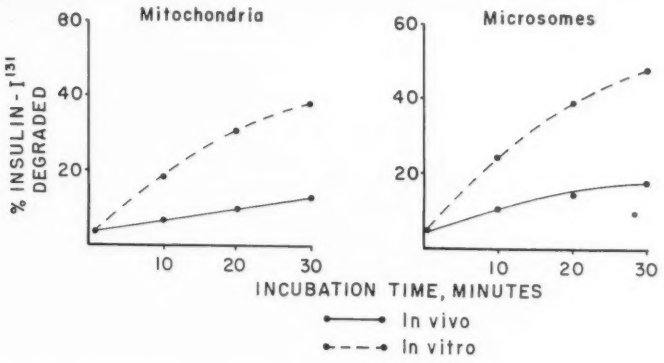

FIG. 4. The degradation of insulin-I^{131} bound to cytostructural element of rat liver: No added "insulinase." Each point is the average of duplicate determinations on 3 different experiments.

soluble I^{131} during particle incubation in the absence of added "insulinase" represents degradation by the non-proteolytic mechanism whereas the values after addition of "insulinase" represent the net effect of both mechanisms.

Figure 4 presents the results of the non-enzymic degradation studies. It is quite apparent that insulin-I^{131} bound to cytoplasmic particles was degraded but to a very much smaller extent than when free in solution, the ratio being approximately 3:1. Similarly, binding resulted in a lesser degradation in those preparations to which "insulinase" was added (Figure 5),

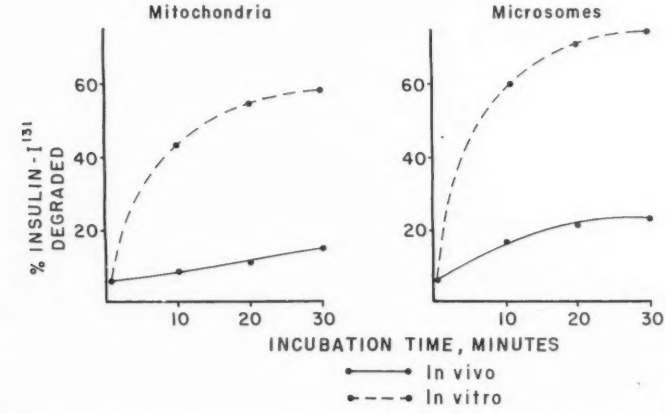

FIG. 5. The degradation of insulin-I^{131} bound to cytostructural elements of rat liver: With added "insulinase." Each point is the average of duplicate determinations on 3 different experiments.

the ratio here being also approximately 3:1. Furthermore, comparison of these curves shows an increment in degradation due to the addition of enzyme for both the *in vivo* and *in vitro* series. The magnitude of this effect of added "insulinase" is shown in Table 2. For mitochondria, addition of "insulinase" to the *in vivo* system resulted in a 2.4% increase in degradation in 10 minutes whereas *in vitro* this value increased over 10 fold. Again, for microsomes the addition of "insulinase" to the *in vivo* system caused only a 6.3% increase in degradation in 10 minutes whereas addition to the

TABLE 2. THE EFFECT OF ADDED "INSULINASE" ON THE DEGRADATION OF INSULIN-I^{131} BOUND TO CYTOSTRUCTURAL ELEMENTS OF RAT LIVER

Twenty ml. beakers, containing 1 ml. of particle suspension (equivalent to 100 mg. of liver) and 0.1 ml. of "insulinase" preparation, were incubated at 37° for 10 minutes. The reaction was stopped with TCA, final concentration of 5%, and the soluble I^{131} expressed in terms of the total I^{131} present. Parallel *in vitro* experiments were conducted in which the amount of added insulin-I^{131} was equal to that determined from the radioassay of the comparable *in vivo* experiment. Each value is the mean of duplicate determinations from 3 independent experiments.

	% Insulin-I^{131} Degraded					
	Mitochondria			Microsomes		
	"Insulinase"		Increment	"Insulinase"		Increment
	+	−		+	−	
In vivo series	8.5	6.1	2.4	17.0	9.7	6.3
In vitro series	44.2	16.8	27.4	59.7	23.2	36.5

in vitro system resulted in a 5-fold greater value. Since, in control experiments, none of these values was changed by increasing the amount of added "insulinase" they may be regarded as reflecting the availability of substrate (insulin-I^{131}) rather than of enzyme.

DISCUSSION

Two distinct avenues of inquiry are relevant to the question of the validity of the intracellular distribution data which provide the basic theme of this report: 1) the specificity of the distribution pattern and 2) the nature of the union of the labeled moiety with the cytostructural element. With regard to the question of specificity, insulin-I^{131} was found to be distributed among the 4 morphologically meaningful fractions of the liver cell in proportions which were both characteristic and unequivocally different from those for insulin-I^{131}, inactivated either with dilute NaOH or extensive iodine substitution (14), human serum albumin-I^{131}, thyroxine-I^{121} and iodide-I^{131} (5). Furthermore, such proportions were distinctly different from those obtained following insulin-I^{131} addition to an homogenate. Secondly, the nature of the union has been investigated in several ways. For one thing perfusion resulted in no removal of insulin-I^{131} from cytostructures whereas the losses ranged from 40% to 65% for inactivated insulin-I^{131} (12), albumin-I^{131}, thyroxine-I^{131} and iodide-I^{131} (5). Also, repeated sedi

mentation (washing) of the cytostructural fractions, in either immediate sequence or with a 2-hour interruption to permit maximal diffusion losses, resulted in little or no removal of insulin-I^{131}; on the other hand, when the labeled hormone had been fixed to cytostructures by non-vital processes (*in vitro experiment*) it was easily, and almost completely, removable. In view of these findings, particularly the last, it seems vain to attempt to support to any significant extent the argument that the distribution pattern resulted from passive phenomena (15). Hence, the previously stated view of the hepatic fate of insulin-I^{131} (5, 14) may be considered to be even more firmly grounded, that insulin-I^{131} penetrates the cell and is bound tightly to mitochondria and microsomes by processes requiring the morphologic integrity of the cell and, by inference, its intact and organized metabolic activities.

The significance of cytostructural binding to the role of insulin in the liver is, however, much less easily stated. For one thing, the view that insulin has a direct and immediate effect on hepatic carbohydrate metabolism has been rather vigorously contested (16) although more recently evidence has been brought forth to make this position somewhat less tenable (17, 18). But even conceding such a direct action, it still cannot be decided whether the observed distribution pattern is a function of the role of insulin in carbohydrate metabolism or of some other physiologically definable aspect such as degradation. These data do provide, however, the basis for an insight into the part played by the liver in the insulin economy of the total organism. Metz has reported, based on pancreatic vein cannulation studies in dogs, that even during severe hypoglycemia insulin continues to be secreted and that an increase in blood sugar of 100 mg.% over normal causes a 2.5 fold increase in the outpouring of the hormone (19). The peripheral effect of this variability may be better appreciated by noting that the value of 54% insulin removal from the blood in a single passage through the human liver is probably a minimal value since, had the same quantity been slowly infused as in the physiologic situation, even more may have been bound to that organ (20). The information presented in Table 2 shows that the bound insulin is relatively unavailable to the degradative activities of the cell, particularly by direct proteolytic intervention, whereas the free hormone is degraded with extreme rapidity. Thus, the major portion of the intracellular insulin, by being bound to cytostructures, is not amenable to removal from the liver and transfer to other sites in the body where it might effect metabolism. Furthermore, once released from the cytostructural binding site in the liver the hormone must be rapidly degraded.

Acknowledgment

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THE EFFECTS OF FASTING AND OF GROWTH HORMONE ADMINISTRATION ON PLASMA FATTY ACID CONCENTRATION IN NORMAL AND HYPOPHYSECTOMIZED RHESUS MONKEYS[1]

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ABSTRACT

The effects of growth hormone and of fasting on the concentration of plasma nonesterified fatty acids (NEFA) in hypophysectomized and intact monkeys (Macaca mulatta) were studied. NEFA concentration was determined by the method of Dole. Small doses (0.05 mg./kg.) of simian growth hormone caused an increase in plasma NEFA concentrations in fasting hypophysectomized and intact monkeys. Bovine and porcine growth hormone preparations were ineffective in this regard as were LTH, TSH, ACTH and Pituitrin. The effect of simian growth hormone was absent or diminished if the animals were allowed access to food during the experiment.

Fasting caused a marked increase in plasma NEFA concentrations within a few hours. Hypophysectomized as well as normal monkeys showed increased NEFA concentrations in response to fasting, suggesting that the NEFA mobilization during fasting is not mediated by an increased secretion of growth hormone by the pituitary gland.

THE demonstration by Lee and Schaffer (1) that growth promoting extracts of anterior pituitary glands administered to rats caused a shift in carcass composition accountable by a decrease in fat and an increase in protein led to numerous studies designed to delineate the role of growth hormone in fat metabolism. Evidence has been adduced which suggests that growth hormone administration produces an elevation in blood lipid concentrations, a mobilization of lipid to the liver, increased ketone body production *in vivo* and *in vitro* and a depression of the R. Q. (cf. 2, 3 and 4). These effects of growth hormone on lipid metabolism coupled with the observation that fat depleted rats failed to grow in response to growth hormone administration led Greenbaum (5) to the postulate that its primary action is on lipid mobilization and oxidation thereby providing the caloric requirements for protein synthesis and growth.

More recently, Raben and Hollenberg (6) have shown that the admin-

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istration of physiological doses of growth hormone markedly increases the concentration of non-esterified fatty acids (NEFA) in the plasma of fasting dogs and human subjects. The metabolic importance of NEFA as a major form of lipid transport and caloric supply has been established by Dole and by Gordon and their co-workers (cf. 7). The above observations led us to an investigation of the action of growth hormone on plasma NEFA levels in the rhesus monkey and of the role of the pituitary gland in the rise of plasma NEFA concentration observed in response to fasting.

MATERIALS AND METHODS

Ten hypophysectomized and 18 normal rhesus monkeys (Macaca mulatta) of both sexes were used in this study. Hypophysectomies (8) were performed at least two months prior to experiment. Completeness of the operation was adjudged by cessation of growth, arrest of sexual development and increase in insulin sensitivity (8). All animals were maintained on a natural diet supplemented by a commercial monkey ration. Water was provided *ad libitum* and, in addition, all hypophysectomized monkeys were provided with a 10% glucose solution (8).

Plasma NEFA concentrations were determined by the method of Dole (9) in 1 ml. samples of plasma derived from heparinized blood obtained by puncture of the femoral vein. In order to minimize possible metabolic effects of repeated venipuncture at least one week was allowed to elapse between experiments performed on any one animal.

All hormones were administered as a single intramuscular injection in aqueous solution[3] one hour following a standardized meal. At the time of injection (0 time of the experiment), remaining food was removed from the cage and, in the case of hypophysectomized animals, the glucose solution was also withheld but tap water was provided *ad libitum* for the remainder of the experiment. This fasting procedure was adopted in an attempt to maintain standardized conditions while minimizing the danger of hypoglycemic coma in the hypophysectomized animals. In the non-fasting situations food was available throughout the experimental period.

Hormone preparations

The simian growth hormone (M 308-15B, M 334B, M 412B, M 425B and M 462B) was prepared by a slight modification of the procedure described by Wilhelmi (10).

Bovine growth hormone (NIH-BGH-1) was the preparation distributed by the Endocrinology Study Section of the National Institutes of Health.

One porcine growth hormone preparation used in these studies was obtained by the method of Raben and Westermeyer (11). The second preparation (P205A) was purified from the oxycel filtrate which is the starting material for the Raben-Westermeyer preparation by a new procedure (12). It is electrophoretically homogeneous. It is similar in molecular weight to monkey growth hormone, has the same N-terminal and C-terminal amino acid (phenylalanine) and it resembles, but is not identical to, monkey growth hormone in amino acid composition. It is less soluble than monkey growth hormone in the isoelectric region (pH 5.5). Biologically, it is equipotent with purified monkey growth hormone and with highly purified bovine growth hormone in the 10 day weight gain test in hypophysectomized rats.

The ovine prolactin (NIH-SP-1) was the material distributed by the Endocrinology

[3] Preliminary experiments have shown that the route of growth hormone administration (I.V. vs. I.M.) does not influence the plasma NEFA response.

Study Section, N.I.H. The thyrotropic hormone (TSH-Armour lot S3112) and corticotropic hormone (ACTH-Upjohn lot 7277) were of bovine origin. The posterior pituitary extract was Pituitrin "S" (Parke, Davis) containing approximately 20 pressor and 20 oxytocic units per ml.

RESULTS

Normal Monkeys

As previously reported in man (9, 13), fasting results in a rapid increase in plasma NEFA concentrations (Fig. 1). This rise in circulating NEFA is fully evident within 4 hours after food is withheld. By 24 hours a 4-fold increase in NEFA concentration can usually be observed. The increase in plasma NEFA concentration in response to fasting appears to be influenced in major fashion by the feeding regimen to which the monkeys are accustomed. Animals fed one meal per day do not exhibit the striking response illustrated in Figure 1 which represents data obtained from monkeys fed twice per day, once in the morning and once in late afternoon.

The administration of simian growth hormone at the beginning of a fast (0 time) markedly increases the plasma NEFA response to the fast. A representative experiment is illustrated in Figure 2A and the mean response

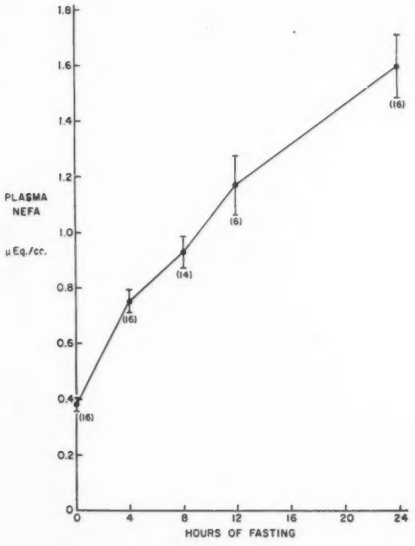

FIG. 1. The effect of fasting on plasma NEFA concentration in intact monkeys. The vertical lines represent standard errors. Numbers in parentheses are the number of animals per point.

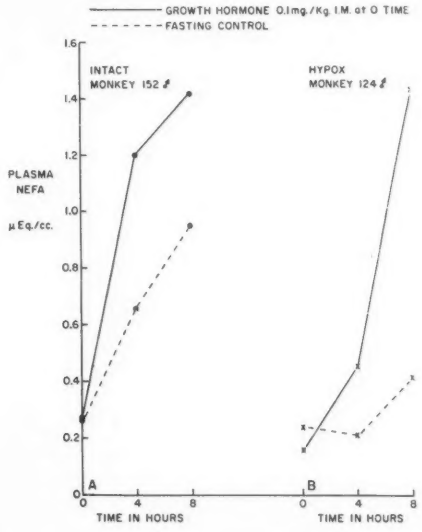

FIG. 2. A. The effect of simian growth hormone on the plasma NEFA concentration in a fasting intact monkey. B. The effect of simian growth hormone on the plasma NEFA concentration in a fasting hypophysectomized monkey.

of a group of animals to 0.05 mg./kg. is summarized in Figure 3A. The minimal effective dose of growth hormone necessary to significantly elevate plasma NEFA concentrations above the control fasting level lies in the range of 0.05–0.1 mg./kg. While a large variability in the response to growth hormone administration has been observed, the response of individual monkeys to a given dose of growth hormone was quite reproducible. A consistent dose-response relationship, however, could not be established.

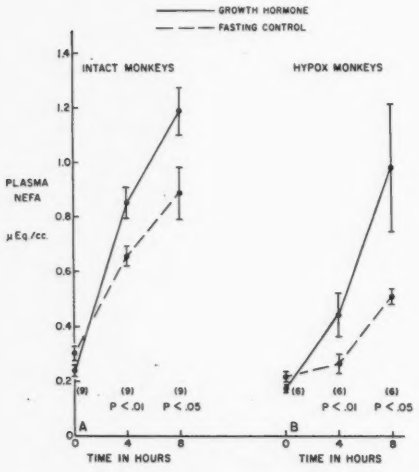

FIG. 3. A. The mean response of a group of fasting intact monkeys to 0.05 mg./kg. of simian growth hormone administered I. M. at 0 time. B. The mean response of a group of fasting hypophysectomized monkeys to 0.1 mg./kg. of simian growth hormone administered I. M. at 0 time.

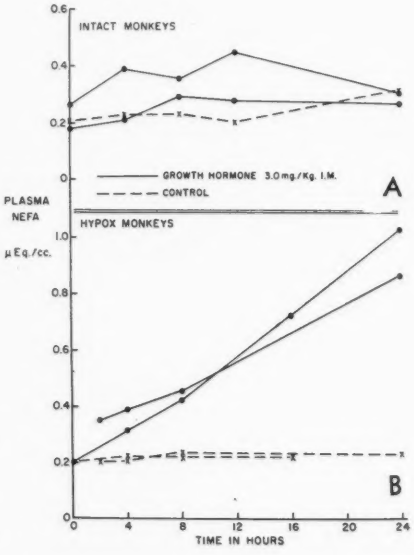

FIG. 4. A. The response of intact monkeys fed *ad lib.* to simian growth hormone. B. The response of hypophysectomized monkeys fed *ad lib.* to simian growth hormone.

Monkeys which exhibited a reduced response to fasting in terms of an increase in plasma NEFA concentration failed to respond to growth hormone administration when the usual experimental procedure was employed. This situation was observed when the animals were on the single daily meal regimen mentioned above. These animals did respond to growth hormone, however, if they were first subjected to an overnight fast.

When intact monkeys were permitted free access to food, simian growth hormone administered at doses 30 to 60 times greater than those found effective in fasting animals failed to effect an increase in plasma NEFA concentrations (Fig. 4).

Hypophysectomized monkeys

Since it has been suggested that growth hormone may be released during fasting (14, 15), it seemed of interest to determine whether the rise in plasma NEFA concentration in response to fasting seen in the normal rhesus monkey is the result of an increased secretion of growth hormone by the pituitary gland. To this end, the fasting responses of hypophysectomized and normal animals were compared. Figures 2B and 3B clearly demonstrate that fasting occasions an increase in plasma NEFA concentration in hypophysectomized monkeys. This increase in the hypophysectomized animals, however, is delayed and not as pronounced as in intact monkeys fasted for the same length of time.

Growth hormone administered to hypophysectomized monkeys at the beginning of a fast significantly increased plasma NEFA concentration above the fasting level. A representative experiment is illustrated in Figure 2B and the response of a group of hypophysectomized monkeys to 0.1 mg./kg. is summarized in Figure 3B. The minimal effective dose of growth hormone in the hypophysectomized animal does not differ from that in intact monkeys albeit the response appears to be less rapid.

In contrast to normal animals, growth hormone administered to hypophysectomized monkeys fed *ad libitum* throughout the experiment did produce an increase in plasma NEFA concentrations (Fig. 4). This increase, however, was considerably smaller than that observed during fasting.

Specificity of the NEFA response to growth hormone

a) *Effect of growth hormone from different species.* Growth hormone preparations of simian, bovine and porcine origin were administered to hypophysectomized monkeys and their effects on fasting plasma NEFA concentrations were compared. A series of representative experiments is illustrated in Figure 5. It may be seen that bovine and porcine growth hormone were ineffective in raising plasma NEFA concentrations above control levels even when administered at 10–80 times the effective dose of simian growth hormone. The porcine preparations were inactive whether administered at acid or alkaline pH.

b) *Effect of other pituitary hormone preparations.* Ovine Prolactin (100 I. U.), bovine ACTH (25 I. U.) and bovine TSH (10 I. U.) were uniformly inactive when administered to hypophysectomized monkeys under conditions which resulted in a marked increase in plasma NEFA concentrations when simian growth hormone was given. A posterior pituitary extract (Pituitrin, 30 U.) was similarly without effect in hypophysectomized animals (Fig. 6). This massive dose, however, did elevate plasma NEFA concentrations above the fasting, control levels in some normal animals, but smaller doses of this preparation were without effect.

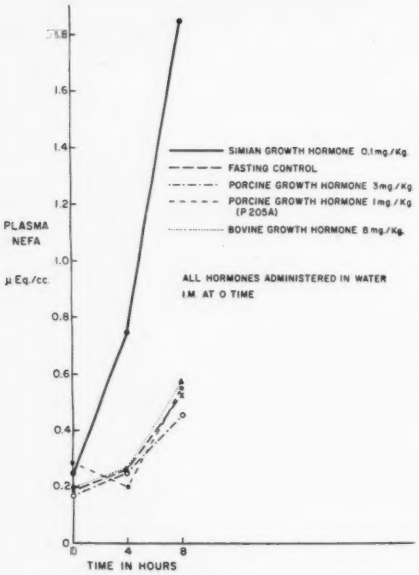
PLASMA NEFA
μ Eq./cc.
SIMIAN GROWTH HORMONE 0.1 mg./Kg.
FASTING CONTROL
PORCINE GROWTH HORMONE 3 mg./Kg.
PORCINE GROWTH HORMONE 1 mg./Kg. (P 205A)
BOVINE GROWTH HORMONE 8 mg./Kg.
ALL HORMONES ADMINISTERED IN WATER I.M. AT O TIME
TIME IN HOURS

FIG. 5. The effects of growth hormones from different species on plasma NEFA concentration in hypophysectomized monkeys.

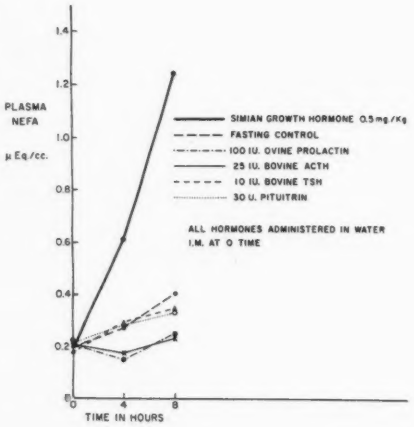

FIG. 6. The effects of various pituitary hormone preparations on plasma NEFA concentration in hypophysectomized monkeys.

DISCUSSION

The observation that growth hormone administration leads to an increase in circulating NEFA strengthens the view that this hormone plays a major role in lipid metabolism. That this effect is due to growth hormone rather than to a contaminant of the preparations used in these experiments is suggested by the following:

a) The "species specific" action of growth hormone on nitrogen retention (16), skeletal growth (17) and carbohydrate metabolism (8) is also demonstrable in the plasma NEFA response. By contrast, bovine, porcine and simian growth hormone all cause an increase in plasma NEFA concentration in the rat (18). b) Small doses of the hormone are required to elicit the effect. c) None of the pituitary preparations tried to date other than growth hormone has produced a significant increase in plasma NEFA concentration in hypophysectomized monkeys.

The sporadic response of intact monkeys to posterior pituitary extract in terms of an increase in plasma NEFA levels merits brief consideration in the light of the recent report by Del Vecchio *et al.* (19) that vasopressin stimulates the secretion of growth hormone in the rat. A similar interpretation could be advanced for the occasional effectiveness of posterior lobe extract in normal monkeys and their invariable ineffectiveness in hypo-

physectomized animals. However, similar experiments performed in the rat (unpublished) have shown that both normal and hypophysectomized animals respond equally to large doses (20 U.) of posterior lobe hormones by an increase in plasma NEFA concentrations but neither responded to lower doses.

Our finding that hypophysectomized animals respond to fasting by an increase in plasma NEFA concentration clearly indicates that this response is not mediated by an increased secretion of growth hormone by the pituitary. It is also evident, however, that the rise in plasma NEFA levels during fasting is not as large nor as rapid in hypophysectomized monkeys as in normal animals. The results of an earlier study (20) which have shown that optimal thyroid function is requisite for the NEFA mobilizing action of epinephrine suggest that the reduced response to fasting seen in hypophysectomized monkeys could be due to the known hypothyroidism of these animals.

The increase in plasma NEFA concentration during fasting is undoubtedly due to an increase in NEFA mobilization from adipose tissue (21, 22). At present, there is no reason to believe that the effect of growth hormone on plasma NEFA levels is not explicable on the same basis. The site and mode of action of growth hormone on NEFA mobilization, however, is not clear. Evidence that growth hormone does not have a direct lipolytic effect on adipose tissue has been obtained by White and Engel (23). That the growth hormone effect is not mediated by a release of epinephrine is suggested by the inability of hypophysectomized monkeys to respond to exogenous epinephrine by an increase in plasma NEFA concentration (20). Since the effect of growth hormone on plasma NEFA concentration in the intact monkey is clearly demonstrable only in the fasting state, it would appear that the action of the hormone "exacerbates" or accentuates the fast. This could be accomplished by further limiting the carbohydrate supply to peripheral tissues or by increasing the metabolic demands on the organism, i.e. by increasing protein synthesis. Should the latter interpretation be correct, NEFA mobilization in response to growth hormone administration would be a phenomenon secondary to the anabolic action of the hormone.

Since the plasma NEFA response to growth hormone in the hypophysectomized rhesus monkey is specific, rapid and easily measured, the use of this animal as an assay animal for the detection of growth hormone preparations suspected of activity in primates is suggested and recommended.

Acknowledgments

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We are grateful to Dr. D. McGinty of Parke, Davis Co. for the gift of several thou-

sand monkey pituitary glands, some of which were used in the preparation of simian monkey growth hormone.

The bovine growth hormone and ovine prolactin were gifts of the Endocrinology Study Section, National Institutes of Health.

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EFFECT OF DI-ETHYL ETHER ANESTHESIA ON THYROID FUNCTION OF RATS: PITUITARY, ADRENAL AND THYROID RELATIONSHIP[1]

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ABSTRACT

The possible role of the pituitary and adrenal hormones in the depressive effect of ether anesthesia on thyroid function in rats was studied. Di-ethyl ether anesthesia for two hours significantly depressed release of radioiodine (I^{131}) from the thiouracil blocked thyroid gland in both bilateral adrenalectomized rats maintained with cortisone and in the bilateral adrenal-medullectomized rats.

Administration of ether anesthesia depressed thyroidal uptake of radioiodine in the hypophysectomized rats treated with small doses of exogenous TSH, although no significant effect was noted in the absence of thyrotropin. Ether anesthesia inhibited the TSH-stimulated release of thyroidal I^{131} in the hypophysectomized rats. Recovery from this inhibition by ether was rapid and more marked when larger amounts of TSH were used.

These findings indicate that the effect of di-ethyl ether anesthesia in rat thyroid was not mediated through the adrenal cortex or medulla. It is suggested that the ether anesthesia might influence thyroid function in rats by inhibiting the action of TSH or by a direct action on the thyroid.

ADMINISTRATION of di-ethyl ether anesthesia has been shown to depress thyroid function in rats as indicated by its effect on thyroidal radioiodine uptake (1), thyroidal release of I^{131} and depression of thyroxine formation (2). Thiopental, another anesthetic, has been reported to suppress the rat thyroid activity directly (3).

The present study was undertaken in an attempt to clarify the possible role of the pituitary and adrenal hormones in the depressive effect of ether anesthesia on thyroid function in rats.

MATERIAL AND METHODS

Bilaterally adrenalectomized rats

Sprague-Dawley strain male albino rats weighing 120–140 gm. were fed Remington's low iodine diet for 5 days prior to operation and throughout the experiment. After bi-

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lateral adrenalectomy, the animals were given 0.9% NaCl water *ad libitum* and cortisone 5 mg. by a single subcutaneous injection daily for 4 days. The last dose of cortisone was injected 12 hours prior to I^{131} injection. Forty hours after intraperitoneal injection of I^{131} (10 μc.), thiouracil was started as described earlier (2). Forty-six hours after I^{131} injection, di-ethyl ether anesthesia was administered for 2 hours. The radioactivity of the neck region was measured by a collimated scintillation counter as described previously (2).

Bilaterally adrenal-medullectomized rats

I^{131} injection, thiouracil and ether anesthesia were given in the same manner to bilaterally adrenal-medullectomized rats. These animals received tap water instead of saline.

Thyroidal release of I^{131} in hypophysectomized rats

Hypophysectomized male albino rats of Sprague-Dawley strain weighing about 100 gm. (purchased from Hormone Assay Laboratories, Chicago, Illinois) were fed Remington's diet and tap water *ad libitum* throughout the experiment. Three days after operation thyrotropic hormone (U.S.P. Thyrotropin Reference Standard, TSH) 0.7 U.S.P. unit diluted with 0.2 ml. of normal saline was given by a single subcutaneous injection every 12 hours for 3 days. The last maintenance injection of TSH (0.7 U.S.P. unit) was given 12 hours after I^{131} (10 μc.) injection. In all groups thiouracil was started 40 hours after I^{131} injection. In one group of animals TSH (0.7 U.S.P. unit) was given at the same time as thiouracil and ether anesthesia (2 hours) was started 3 hours later. In another group, a larger dose (1.4 U.S.P. units) of TSH was used and in another group no TSH, other than the maintenance TSH, was given. In this latter group, 31 hours elapsed after the final TSH injection before ether anesthesia was administered.

Thyroidal uptake of I^{131} in hypophysectomized rats

In another group of hypophysectomized rats maintained with TSH in the same manner for 3 days, I^{131} (1 μc.) and ether anesthesia for 2 hours were given 8 hours after injection of the last TSH (0.7 U.S.P. unit). Some hypophysectomized rats, who received no TSH throughout the experiment, were given 10 μc. of I^{131} and ether for 2 hours as above. The radioactivity of the dissected thyroid gland was measured in a well type scintillation counter.

RESULTS

Adrenal cortex and thyroid

Ether anesthesia significantly depressed I^{131} release from the blocked thyroid in the bilateral adrenalectomized rats maintained with cortisone (Table 1).

Adrenal medulla and thyroid

Administration of ether anesthesia for 2 hours diminished I^{131} release from the blocked thyroid in the bilaterally adrenal-medullectomized rats (Table 1).

Pituitary and thyroid

Ether anesthesia significantly reduced thyroidal uptake of I^{131} in the hypophysectomized rats treated with TSH, but showed no significant

TABLE 1. EFFECT OF DI-ETHYL ETHER ANESTHESIA FOR 2 HOURS ON THYROIDAL I^{131} SECRETION IN BILATERAL ADRENALECTOMIZED AND ADRENAL-MEDULLECTOMIZED RATS

Interval after "zero time"*	Control		Ether	
	I^{131} remaining in thyroid		I^{131} remaining in thyroid	
Adrenalectomized rats				
0	100	(16)†	100	(15)
6 hours	76.5 ± 1.8‡		89.0 ± 1.2	(t = 5.5 P < 0.001)
Adrenal-medullectomized rats				
0	100	(17)	100	(21)
6 hours	84.4 ± 2.3		90.3 ± 0.9	P < 0.02)

* The "zero time" count was made 46 hours after I-131 injection and 6 hours after thiouracil. Anesthesia started from "zero time."
† Number of rats ( ).
‡ Mean ± standard error.

effect without TSH (Table 2). I^{131} release from the blocked thyroid in the hypophysectomized rats maintained with TSH was not significantly influenced by ether anesthesia 31 hours after the last injection of TSH. Ether anesthesia, however, inhibited thyroidal release of I^{131} during the course of anesthesia in both groups of animals which received 0.7 and 1.4 U.S.P. units of TSH 3 hours before ether was given. Ether anesthesia still depressed glandular release of I^{131} 4 hours after anesthesia in the former group, but had no effect in the latter group (Table 3 and Fig. 1).

DISCUSSION

Evidence concerning the role of the adrenal cortex in thyroid activity is conflicting. Some workers have reported a diminution of thyroidal release of radioiodine by cortisone in man and in the animal, suggesting that cortisone inhibits thyroid function by suppressing hypophyseal secretion of thyrotropin (4, 5, 6, 7). On the contrary, Beck (8) has reported an increase of

TABLE 2. EFFECT OF 2-HOUR DI-ETHYL ETHER ANESTHESIA ON 2-HOUR THYROID UPTAKE OF I-131 IN HYPOPHYSECTOMIZED RATS

Group	No. of rats	Body wt. (gm.)	% of administered I^{131} in thyroid	
No TSH				
Control	10	103 ± 4*	0.3 ± 0.01* (100%)	t = 0.9
Ether	10	107 ± 6	0.2 ± 0.01 (67%)	P < 0.40
With TSH				
Control	12	95 ± 5	16.1 ± 1.2 (100%)	t = 5.4
Ether	11	94 ± 7	8.1 ± 0.6 (50%)	P < 0.001

* Mean ± standard error.

TABLE 3. EFFECT OF DI-ETHYL ETHER ANESTHESIA FOR 2 HOURS ON THYROIDAL RELEASE OF I-131 IN HYPOPHYSECTOMIZED RATS

Interval after "zero time"*	Control		Ether	
	I^{131} remaining in thyroid		I^{131} remaining in thyroid	
No TSH injection				
0	100	(11)†	100	(11)
6 hours	96.6 ± 1.6‡		97.3 ± 1.7	
TSH (0.7 U.S.P. units)				
0	100	(16)	100	(16)
6 hours	85.5 ± 3.7		91.5 ± 3.7	(t = 4.7 P < 0.001)
TSH (1.4 U.S.P. units)				
0	100	(9)	100	(11)
6 hours	76.2 ± 5.9		76.0 ± 4.2	

* The "zero time" count was made 43 hours after I^{131} injection and 3 hours after TSH and thiouracil injection. Anesthesia started from "zero time."
† Number of rats ( ).
‡ Mean ± standard deviation.

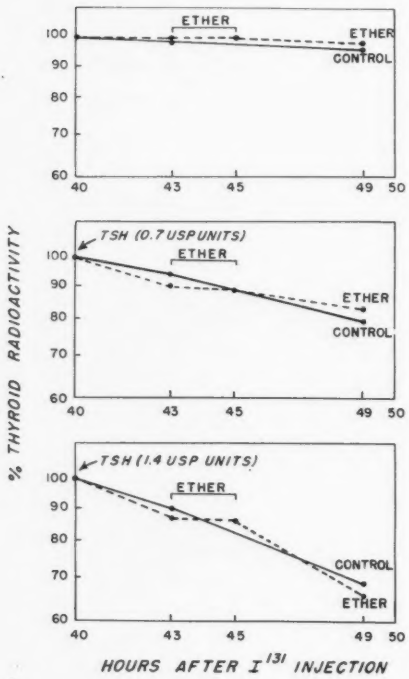

FIG. 1. Effect of di-ethyl ether anesthesia for 2 hours on thyroidal release of I^{131} in the hypophysectomized rats.

Note: TSH (0.7 U.S.P. unit) was given 31 hours prior to ether anesthesia in the top figure. Ether anesthesia significantly depressed I^{131} release in the hypophysectomized rats receiving TSH (0.7 U.S.P. unit) 3 hours prior to anesthesia. However, its effect was transient in the rats receiving a larger dose of TSH (1.4 U.S.P. units).

glandular release of I^{131} by cortisone in the intact rat. Other workers, however, have failed to find any effect of cortisone on the rate of I^{131} diminution in the unblocked thyroid in animals (9, 10, 11, 12).

Administration of anesthesia, like other stresses, has been shown to increase blood concentration of 17-hydroxycorticoid (13, 14). This increase in adrenal activity might have accounted for the previously observed (2) decrease in thyroidal activity in rats. The present study demonstrated that ether anesthesia reduced thyroidal release of I^{131} in the presence or absence of the adrenal cortex. These findings would indicate that the effect of ether anesthesia on thyroid function in rats was probably not mediated through the adrenal cortex.

The role of epinephrine on thyroid activity is not well understood. Some workers have reported that the administration of epinephrine resulted in a decrease of thyroid uptake of I^{131} (15, 16, 17, 18). On the other hand, others have reported that the administration of epinephrine increased thyroidal uptake of I^{131} in man (19) and increased thyroidal release of PBI-131 in the dog (12). Soderberg (11) observed that an increase or decrease of glandular release of I^{131} in the dog and cat occurred and was dependent on the dose of epinephrine or norepinephrine. Harris (4) and Brown-Grant and Gibson (17), on the other hand, doubted that the release of epinephrine in stress modified thyroid activity.

It is known that ether anesthesia increases the blood concentration of epinephrine or norepinephrine in man and in the animal (20–22). It was, therefore, desirable to investigate a possible influence of the adrenal-medulla in depressing the thyroid activity caused by ether anesthesia in rats. In the present study ether anesthesia depressed thyroidal release of I^{131} in the presence or absence of the adrenal-medulla. This indicates that the medulla was not associated with suppression of thyroidal activity caused by ether anesthesia.

Since it has been shown that thyrotropin stimulates the release of iodine from the thyroid (23), an effect of ether on the pituitary or a higher center could account for the observed decrease in release of radioiodine. In hypophysectomized rats given thyrotropin (0.7 and 1.4 U.S.P. units 3 hours before anesthesia) ether still decreased the release of radioiodine. The lack of an effect when ether anesthesia was given 31 hours after the last dose of thyrotropin can be explained by thyroid activity being so low that any further slowing of release could not be measured. Thyrotropin may have reached very low blood levels by 31 hours even in hypophysectomized animals (24).

The return to a faster release rate of radioiodine after slowing by ether anesthesia when 1.4 U.S.P. units of TSH had been used was presumably related to the larger dose overcoming the residual inhibition of ether. These observations indicate that ether anesthesia can change the thyroidal

release of I^{131} in the absence of the pituitary but exogenous thyrotropin is needed to maintain thyroidal activity so that such a change can be measured. Similarly, the uptake of radioiodine was decreased by ether anesthesia in hypophysectomized animals who had received TSH, but no effect was noted in those animals not given TSH. The above explanation would seem to apply to this observation.

The evidence presented suggests, then, that ether anesthesia inhibits thyroidal activity either by acting against thyrotropin or by acting directly on the thyroid. The possible changes in the proportions of intrathyroidal iodinated compounds previously noted (2) do not help in deciding which of these alterations is correct, since an inactivation of thyroxine would tend to lower the thyronine content relative to the iodinated tyrosines (25). Preliminary *in vitro* studies have not shown any significant effect of ether on the uptake of radioiodine by thyroid slices. This, evidence, although not conclusive, suggests that an action against thyrotropin by ether may be the more likely explanation.

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STEROID HYDROXYLATION IN HUMAN FETAL ADRENALS[1]

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ABSTRACT

Homogenates of human fetal adrenals were incubated *in vitro* with a TPNH generating system and progesterone or 17α-hydroxyprogesterone as substrate. At all ages tested the adrenals possessed the ability to convert progesterone to Porter-Silber chromogens having the chromatographic properties of cortisol. In addition, incubations with adrenal slices revealed the presence of an active hexosemonophosphate shunt, which could provide TPNH for the steroid hydroxylations. There was no difference with age in hydroxylation per mg. adrenal tissue; however, as adrenal gland size increased the amount of cortisol formed per adrenal increased.

SEVERAL different methods have been used to study the function of the human fetal adrenal. Gardner *et al.* (1) measured the amount of 17-ketosteroids in umbilical cord blood and in maternal blood at parturition and found a higher level of 17-ketosteroids in the cord blood. Migeon *et al.* (2) have compared maternal and fetal plasma levels of 17-hydroxycorticosteroids. Their data suggest that 17-hydroxycorticosteroids pass from maternal to fetal circulation in a fixed ratio. Both 17-ketosteroids and 17-hydroxycorticosteroids in the plasma of the newborn fall to nearly zero within a short time after birth.

Bloch and Benirschke (3) made extracts of human fetal adrenals obtained at therapeutic abortions. By paper chromatography they characterized dehydroepiandrosterone, androstenedione, and 11β-hydroxyandrostenedione in these extracts, but could find no 17-hydroxycorticosteroids prior to about the twentieth week of gestation. Later, however, trace amounts were found.

Solomon *et al.* (4) incubated human fetal adrenal tissue *in vitro* with progesterone-4-C^{14}. Fetal zone was dissected from adult zone, homogenized and incubated separately. By pooling the extracts from 26 pairs of adrenal glands they obtained enough material to identify products by chemical

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means. 17α-hydroxyprogesterone and Δ^4-androstenedione were formed both in the fetal zone and adult zone incubations. Lanman and Silverman (5) had previously provided evidence that adrenals of newborn infants could convert progesterone into cortisol and corticosterone.

This paper presents the results of experiments designed to determine whether or not the human fetal adrenal, at various stages of gestation, possesses the hydroxylating enzymes needed to convert progesterone to cortisol.

Some of the previous studies suggested that this gland is unable to synthesize cortisol in significant amounts. It was, therefore, of interest to investigate the hydroxylases of the adrenal at different times in development. An excess of substrate and known cofactors was supplied in order that the amount of enzyme would presumably be the rate limiting factor in product formation.

In addition to assaying the enzymes themselves, we studied the general pathways of metabolism in the adrenal in order to determine whether these reactions could provide the necessary cofactors and energy requirements for steroid hydroxylation.

MATERIALS AND METHODS

Human fetal adrenals were obtained immediately at therapeutic absorptions and homogenized in 5 ml. of phosphate buffer at pH 7.4 containing 73.4 μmoles K^+, 19.2 μmoles Mg^{++}, 38.4 μmoles Cl^-, and 40.0 μmoles $PO_4^{=}$. Aliquots of the homogenate equivalent to 200 to 400 mg. of tissue were transferred to incubation vessels and a steroid substrate added. All steroids were dissolved in either absolute ethanol (1 mg./ml.) or in propylene glycol (1 mg./ml.). Triphosphopyridine nucleotide (2.7 μmoles) and glucose-6-phosphate (24 μmoles) were added as a TPNH-generating system. In selected experiments, oxygen consumption was measured in a Warburg respirometer. At the end of the incubation period, an aliquot of the incubation material was removed for Porter-Silber (6) analysis, and the remainder was frozen for subsequent paper chromatography. The absorbance was measured in a Unicam spectrophotometer over a range of wavelengths from 360 to 500 mμ. With each set of determinations, a standard curve using cortisol was measured over the same range. Only substances with a peak at or very near 410 mμ were considered to be Porter-Silber chromogens.

In other experiments, slices of adrenals were incubated with various radioactive substrates (glucose-1-C^{14}, glucose-6-C^{14}, pyruvate-1-C^{14}, pyruvate-2-C^{14}, and acetate-2-C^{14}) in a Warburg respirometer. Oxygen consumption, carbon dioxide production, substrate utilization (7, 8), lipid (9) and lactate (10) formation were measured. Radioactive products were isolated (11), recrystallized and the specific activity of the material determined in a windowless, gas-flow proportional counter. Each sample was counted long enough to reduce the probable error of counting to less than 3% and corrected for self absorption (12).

Aliquots for paper chromatography were extracted three times with seven volumes of acetone, followed by three times with seven volumes of ethyl acetate. The extracts were pooled, dried on a rotating still, and partitioned between hexane and 90% methanol using a five funnel counter-current distribution with stripping (13). The methanol extract was dried and put through a five funnel counter-current distribution with stripping using ethyl acetate and water as the solvents.

An aliquot of the ethyl acetate extract was transferred to Whatman #2 filter paper and developed in a Bush system for chromatography (14). In all instances either the toluene 90:ethyl-acetate 10/methanol 60:water 40 system or the ligroin 2:toluene 1/methanol 7:water 3 system was used. Occasionally the Zaffaroni (15) toluene/propylene glycol system was used in addition. Papers were scanned under ultraviolet light and then sprayed with either the blue tetrazolium (16) or Zimmerman (17) reagent.

RESULTS

Progesterone. Adrenals incubated with progesterone as substrate yielded Porter-Silber chromogens in all cases but the very youngest (Table 1). Extraction of adrenals before incubation yielded no chromogen, giving further evidence that the material was actually formed during incubation. There is no statistically significant relation between age and the amount of chromogen formed per mg. tissue. There is, however, an increase in chromogen made per adrenal as gland size increases.

The amounts of Porter-Silber chromogen formed with and without cofactors are shown in Table 2. It is apparent that the addition of TPN and glucose-6-phosphate greatly increases the yield. The apparent depressing effect of ascorbic acid on chromogen formation is probably not significant.

In four of the progesterone experiments, oxygen comsumption of the adrenal homogenates was measured. The values were 14, 15, 17, and 20.3 μmoles oxygen consumed per gram tissue and hour in the presence of TPN and glucose-6-phosphate. In the absence of cofactors (one experiment) the value was 3.4 μmoles per gram tissue and hour.

TABLE 1. HYDROXYLATION OF PROGESTERONE BY HUMAN FETAL ADRENALS

Sex	Fetal wt. (gm.)	Crown-rump (cm.)	Gestational age (wk.)	Adrenal wt. (mg.)	μmole F*/gm. tissue and hour
?	35	6.5	?	95	0
?	35	8.0	11.5	102	.200
?	38	8.0	11.0	122	.303
F	75	11.5	13.0	431	.050
F	112	11.5	16.0	411	.110
M	185	13.0	19.0	835	.207
M	210	14.5	17.0	773	.353
M	270	14.5	16.5	1,042	.240
M	430	15.5	?	564	.292
F	250	16.5	18.0	1,377	.132
					.138
M	315	16.5	17.0	1,161	.333
F	?	18.0	18.0	1,274	.402
F	500	18.0	24.0	1,672	.100
M	?	19.0	20.0	1,741	.116
F	?	20.5	21.0	1,180	.231
M	1,385	25.5	?	3,564	.085
					.194 ± .0088†

* The Porter-Silber chromogens produced are all expressed in terms of micromoles of cortisol (F) used as standard.

† Mean ± standard error.

Adrenal homogenates incubated with 0.80 μmoles progesterone, 24 μmoles glucose-6-phosphate, and 2.7 μmoles TPN in phosphate buffer at pH 7.4 and 37° C.

Paper chromatography of extracts of four different adrenals revealed, in each case, the presence of an ultraviolet absorbing, blue tetrazolium reducing substance, with an R_f in ligroin:toluene/70% methanol identical with that of cortisol. Another substance was present, with the following properties: ultraviolet absorbing, blue tetrazolium reducing, Zimmerman negative, and an R_f of 0.66 in ligroin:toluene/70% methanol and an R_c (with reference to corticosterone in the same system) in Zaffaroni's toluene /propylene glycol of 0.88. The amounts of cortisol and compound X do not permit further characterization at the present time. However, other ex-

TABLE 2. EFFECT OF COFACTORS ON HYDROXYLATION OF STEROIDS BY FETAL ADRENALS

Sex	Fetal wt. (gm.)	Crown-rump (cm.)	Gestational age (wk.)	Substrate	μmoles F/gm. tissue and hour	Cofactors
?	235	15.5	17	Progesterone	.044	None
					.120	2.7 μmoles TPN
M	185	13.0	19	Progesterone	.047	None
					.121	2.7 μmoles TPN
					.206	2.7 μmoles TPN + 24 μmoles Gluc-6-PO_4
F	660	20.0	?	Progesterone	.138	1.4 μmoles TPN + 12 μmoles Gluc-6-PO_4
					.107	1.4 μmoles TPN + 12 μmoles Gluc-6-PO_4 + .512 μmoles ascorbic acid
F	660	20		17 OH Prog.	.460	1.4 μmoles TPN + 12 μmoles Gluc-6-PO_4
					.424	1.4 μmoles TPN + 12 μmoles Gluc-6-PO_4 + .512 μmoles ascorbic acid

Adrenal homogenates incubated with .80 μmoles of progesterone or .76 μmoles 17α-hydroxyprogesterone, with cofactors as indicated, in phosphate buffer at 37° C.

periments with radioactive progesterone may make possible the chemical identification of these products.

17α-hydroxyprogesterone. In all cases, adrenals incubated with 17α-hydroxyprogesterone produced more Porter-Silber chromogen per gram tissue an hour than those adrenals incubated with progesterone (Table 3). Figure 1 presents the data of those experiments in which aliquots of the same adrenal homogenate were incubated with progesterone and with 17α-hydroxyprogesterone.

In several experiments partial fractionation of the adrenal homogenates was performed before incubation. The adrenals were homogenized in 0.25 *M* sucrose to make a 20% homogenate and the homogenate was centrifuged at 5° C and 1500 g. for 20 minutes to remove nuclei and cellular

TABLE 3. HYDROXYLATION OF 17α-HYDROXYPROGESTERONE BY HUMAN FETAL ADRENALS

Sex	Fetal wt. (gm.)	Crown-rump (cm.)	Gestational age (wk.)	Adrenal wt. (mg.)	μmoles F/gm. tissue and hour
M	35	8.5	12	160	.698
F	112	11.5	16	411	.443
M	?	12.0	15.5	316	.304
F	180	14.0	16	828	.352
M	170	14.5	16	782	.392
M	430	15.5	?	564	.314
M	315	16.5	17	1,161	.646
F	?	18.0	18	1,274	.738
F	500	18.0	24	1,672	.295
F	675	20.0	21	?	.246
M	1,385	25.5	?	3,564	.361
					.435 ± .053

Adrenal homogenates incubated with 0.76 μmoles 17α-hydroxyprogesterone, 24 μmoles glucose-6-phosphate, and 2.7 μmoles TPN in phosphate buffer at pH 7.4 and 37° C.

debris. Thereafter, the supernatant fluid was transferred to a clean tube and centrifuged at 12,000 g. for 30 min. to sediment the mitochondria. The supernatant fluid was removed and the mitochondria were washed twice with 0.25 *M* sucrose. The washed mitochondria and the supernatant fluid were incubated in paired vessels in phosphate buffer at pH 7.4 with 17α-hydroxyprogesterone as substrate. A TPNH generating system was added. After two hours of incubation at 37° C an aliquot was removed and analyzed for Porter-Silber chromogen. The results are summarized in Table 4. The formation of Porter-Silber chromogen from 17α-hydroxy progesterone demonstrates the presence of the enzyme, 21-hydroxylase. It can be seen that roughly five times more activity was found in the mitochondria than could be demonstrated in the supernatant fluid (which contains the microsomes). This is in contrast to previous studies with beef adrenal in which 21-hydroxylase was located largely in the microsomes (18).

Paper chromatography of the extracts from two adrenals incubated

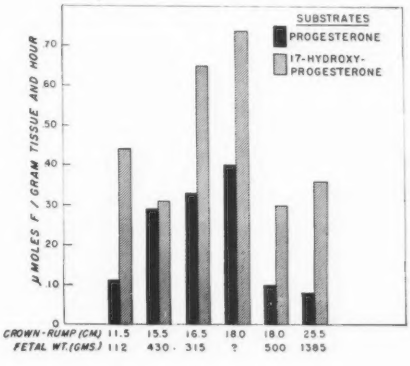

FIG. 1. Comparison of Progesterone and 17α-hydroxyprogesterone as substrates for steroid hydroxylation. Each pair of bars represents the results of incubations with aliquots of a single adrenal homogenate.

with 17α-hydroxyprogesterone again demonstrated the production of cortisol and an unknown compound X, in roughly equal amounts.

Corticosterone and Desoxycorticosterone. Attempts to demonstrate the conversion of either corticosterone or desoxycorticosterone to Porter-Silber chromogen were, for the most part, unsuccessful. In four such experiments no significant amount of chromogen was formed.

Acetate, Cholesterol, Pregnenolone (3β-hydroxypregn-5-en-20-one). Adrenals from fetuses having a crown-rump length of 10 to 25 cm. were in-

TABLE 4. HYDROXYLATION OF 17α-HYDROXYPROGESTERONE BY MITOCHONDRIA AND SUPERNATANT FLUID OF FETAL ADRENAL HOMOGENATES

Sex	Crown-Rump (cm.)	Gestational (wk.) age	μmoles F*/gm. tissue and hour	
			mitochondria	supernatant fluid
F	15.0	17	.180	.092
F	12.5	15	.264	.072
M	12.0	14	.306	.055
F	16.5	?	.375	0

* Micromoles of F are calculated in terms of mitochondria or supernatant fluid equivalent to (or obtained from) so many grams of adrenal tissue.

Mitochondria and supernatant fluid incubated with 0.76 μmoles 17α-hydroxyprogesterone, 24 μmoles glucose-6-phosphate, 2.7 μmoles TPN in phosphate buffer at pH 7.4 and 37° C.

cubated with either cholesterol or pregnenolone as substrate. In all instances a TPNH generating system was added and in many experiments DPN was also included. No conversion of cholesterol to Porter-Silber chromogen was found in nine such incubations. In three additional experiments with cholesterol, the presence of ACTH in the incubation medium had no observable effect. No ultraviolet absorbing material was found in paper chromatography of extracts of several of these incubation mixtures, thus indicating that no Δ^4-3-ketosteroids has been formed. However, incubation of adrenals from two older fetuses yielded material reacting with Zimmerman reagent on paper. The R_f of this material in the ligroin:toluene/70% methanol system was identical with that of dehydroepiandrosterone.

Using pregnenolone as substrate small amounts of Porter-Siliber chromogen were formed in only two of ten experiments (0.0073 μg./mg. tissue and 0.0182 μg./mg. tissue). Both experiments were with older fetuses (500 gm.—18 cm. and 1385 gm.—25.5 cm.) and involved pooling the extracts of three separate incubation vessels. Again the addition of ACTH was without effect. Paper chromatography of the extract from an incubation with the adrenal of 675 gm. fetus revealed a small amount of what may have been cortisol.

Two experiments with acetate as substrate showed no Porter-Silber chromogen formation.

Non steroid substrates. With glucose as substrate the fetal adrenal has a

average R.Q. of 1.75 (Table 5). The complete oxidation of glucose is associated with an R.Q. of 1.00. The R.Q. of 1.75 found in these experiments indicates a rapid rate of lipogenesis. In agreement with this observation is the figure for conversion of substrate to lipid, 0.126 μmoles per gram tissue and hour. The value is similar to that found for fetal liver, and is considerably larger than that found in other fetal organs. Both liver and adrenal thus show very active lipogenesis.

In comparing the amount of labelled carbon dioxide from glucose-6-C^{14}

TABLE 5. METABOLIC DATA ON HUMAN FETAL ADRENALS INCUBATED WITH RADIOACTIVE GLUCOSE

Fetal wt. (gm.)	Crown-rump (cm.)	Subst.	O_2 cons.	CO_2 prod.	R.Q.	Lactate prod.	Subst. to CO_2	Subst. to lipid
430	15.5	G^1	15.6	27.5	1.31	43.5	.860	.126
		G^6	16.2	21.1	1.30	38.5	.042	
?	18.0	G^1	18.9	31.9	1.69		.575	
		G^6	17.1	31.1	1.82		.044	
		G^1	25.2	28.9	1.14		1.318	
		G^6	22.3	45.0	2.02		.106	
		G^1	22.8	41.2	1.81		1.432	
		G^6	18.2	26.8	1.47		.053	
?	18.0	G^1	26.6	42.7	1.60	18.7	.630	
		G^6	23.9	81.4	3.40	14.4	.068	
675	20.0	G^1	33.3	65.6	1.97	38.8	3.270	G^1—.137
		G^6	25.5	56.0	2.20	43.1	.100	G^6—.127
		G^1	18.5	38.2	2.06	53.2	2.650	
		G^6	25.4	46.4	1.83	36.6	.088	
750	*	G^1	12.7	23.1	1.82		.476	
		G^6	10.6	11.2	1.06		.032	
		G^1	4.3	16.8	3.85		.160	
		G^6	13.1	9.0	.69		.004	

* Premature infant who lived for two hours. The tissue was obtained two hours after death.

All values are expressed in micromoles per gram adrenal per hour.

Slices of fetal adrenals incubated with 16.5 μmoles of either glucose-1-C^{14} or glucose-6-C^{14} in buffer containing 80 μmoles Na, 40 μmoles K, 10 μmoles Mg, 70 μmoles PO_4 and 60 μmoles Cl, at pH 7.4 and 37° C.

with that from glucose-1-C^{14}, we obtained an average G6/G1 ratio of 0.056. If one assumes that carbon 6 of glucose is converted to CO_2 only via the glycolytic cycle, whereas carbon one may be metabolized to CO_2 either by way of the glycolytic cycle or the hexose monophosphate shunt, it is possible to obtain an estimate of the fraction of glucose metabolized by each pathway. The G6/G1 ratio of 0.056 would indicate a very active hexose monophosphate shunt. This is by far the lowest ratio we have seen in fetal tissues. For comparison the following values were obtained (19): liver 0.42, lung 1.0, heart 0.37, cerebral cortex 0.93. The first two steps of the hexose monophosphate shunt involve the conversion of TPN to TPNH and thus provide an active TPNH generating system. TPNH is known to

be a necessary cofactor for both lipid synthesis (20), and steroid hydroxylation (21). Thus by being linked with lipogenesis, the TPNH generated in the hexose monophosphate shunt could be reoxidized to TPN.

Another interesting aspect of the glucose experiments is the relatively high rate of lactate formation by the fetal adrenal (average 35.8 μmoles per gram tissue and hour). With either acetate or pyruvate as substrate, the value is much lower (approximately 10 μmoles lactate formed per gram tissue and hour).

With pyruvate-1-C^{14} as substrate the fetal adrenal converted 0.022 μmoles pyruvate to lipid per gram tissue and hour. The pyruvate-2-C^{14} experiments gave a value of 0.399. In contrast to this, in the conversion of pyruvate to CO_2, carbon one is a better source than carbon 2 (pyruvate-1-C^{14} yielded 6.1 μmoles CO_2, pyruvate-2-C^{14} yielded 1.8 μmoles). Since carbon one of pyruvate (the carboxyl carbon) is lost in the formation of acetyl coenzyme A one would expect this carbon to appear as CO_2 and to have little or no part in lipid synthesis. Carbon two (carbonyl carbon) however, would remain an integral part of acetyl CoA and participate in lipogenesis.

DISCUSSION

Though absolute identification of cortisol has not been completed, these data and those of Lanman (5) suggest strongly that the fetal and newborn adrenal can synthesize cortisol using progesterone as substrate. As additional evidence we have analyzed by column chromatography extracts from an adrenal incubated with progesterone-4-C^{14} and obtained labelled cortisol (characterized by paper chromatography) with constant specific activity after repeated recrystallizations. Thus it would appear that the human fetal adrenal possesses the enzymes for hydroxylating progesterone at positions 17, 21 and 11. The presence of an unknown rather polar Δ^4-3-ketone with an alpha ketol group on the side chain raises the possibility of still other hydroxylase enzymes. For maximum activity of these enzymes a TPNH generating system was necessary.

The data on the hexose monophosphate shunt activity of the adrenal reveals that a very active TPNH generating pathway is indeed present. Since glucose is readily available as a substrate for the metabolism of the adrenal, it seems quite unlikely that shunt activity *in vivo* could be limited by insufficient substrate. Thus with a highly active glucose-6-phosphate dehydrogenase system and glucose as substrate, one may conclude that the adrenal probably is able to generate large quantities of TPNH *in vivo* as well as *in vitro*. One may further postulate, that if the adrenal were supplied with a source of progesterone, and with sufficient TPNH, it could synthesize cortisol. One could visualize it as a coupled enzyme system as in Figure 2.

Our inability to demonstrate a conversion of pregnenolone to cortisol

might suggest either a lack or an extreme lability of the enzyme 3β-ol dehydrogenase. Present knowledge of this enzyme would indicate that DPN is a necessary cofactor (22). Supplying this cofactor, however, did not produce the expected conversion except in two fetuses relatively late in gestation. It is impossible to state, without further study, whether there is indeed a lack or inactivity of the enzyme required for the conversion of pregnenolone to progesterone.

These experiments provide two additional items of interest regarding hydroxylases. First, our data would indicate that the 21-hydroxylase is to

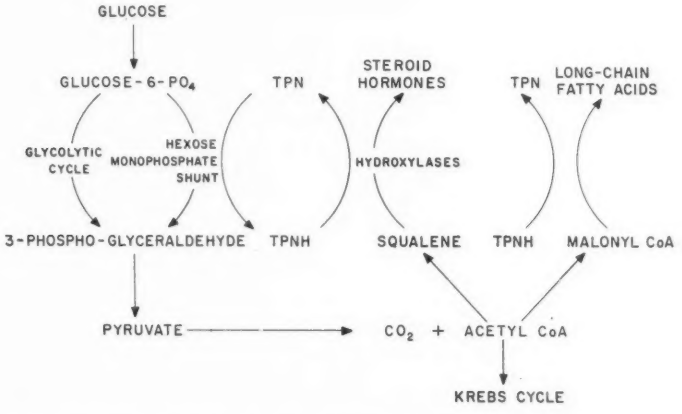

FIG. 2. A hypothetical scheme for the coupling of hexosemonophosphate shunt with steroid hydroxylation and with fatty acid synthesis.

be found largely in the mitochondria (or, more specifically, the material sedimented at 12,000 g.) rather than in the microsomes. This might be simply a species difference (man vs. cow) or it might conceivably be a difference between fetal and adult tissue. Slaunwhite (23) has reported the presence of 21-hydroxylase of an adult human adrenal in the microsomal fraction. It would be of interest to investigate this difference further.

The second interesting finding is the apparent order of hydroxylation. Progesterone is readily hydroxylated at either carbon 17 or 21 to produce 17α-hydroxyprogesterone or corticosterone. However, once carbon 21 is hydroxylated, i.e. using corticosterone or desoxycorticosterone as substrate, further hydroxylation at carbon 17 does not seem to occur to any measurable extent. Similar results were obtained by Hechter (24) in beef adrenal perfusions.

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EFFECTS OF HORMONES ON GLUCOSE UTILIZATION IN FASTED RATS[1]

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ABSTRACT

The permeability of tissues toward glucose was explored in rats under various hormonal and environmental influences. The velocity constant K expressing the rate of disappearance of glucose from blood after rapid intravenous injection of a small load of glucose was critically analyzed as an index of glucose utilization. Acute treatments with large doses of glucagon and hydrocortisone led only to negligible effects on K. One hour after epinephrine injection, the persisting hyperglycemia appeared to be due more to increased liver glycogenolysis than to inhibition of glucose utilization. A marked enhancement of K developed 2 hours after a moderate dose. As expected, severe insulin hypoglycemia was accompanied by a sharp increase in K; this was followed by a drastic decrease during the restoration to normal of the blood glucose level. A single injection of thyroxin led to a rapid increase in K and to a small degree of hyperglycemia. After pretreatment of the animals with thyroxine, the moderate hyperglycemia persisted but K returned to normal. Rats exposed to 5° C for 4 hours exhibited a small decrease in K. When cold exposure was prolonged for 14 days, an inverse reaction was noted, together with marked insulin hypersensitivity.

BLOOD glucose levels decrease exponentially with time after the rapid intravenous injection of a small load of glucose. The velocity constant (K) of the rate of glucose disappearance can be calculated from the formula:

$$G = G_0 e^{-Kt}$$

where G is the glucose concentration in mg.% at time t measured in minutes. K is expressed as the percentage of blood glucose decrease per minute and is determined by plotting all the experimental values on a semilogarithmic scale and measuring the slope of the best-fitting straight line. K does not vary when the test is repeated and can be considered, with some restrictions, as a good index of the permeability of tissues toward glucose (1).

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Unlike what can be done with the usual modes of expression of glucose tolerance, the rate of glucose utilization can be expressed by a single number and is therefore readily amenable to statistical analysis. It was therefore felt useful to explore the effects in rats of various hormones on K with particular emphasis being put on the doses and the duration of the treatment. Results obtained after acute treatment with insulin and growth hormone and chronic treatment with growth hormone and cortisone have already been published (2, 3, 4). In the present study K was measured at various times following single intraperitoneal injections of epinephrine, glucagon, insulin, hydrocortisone and thyroxine (the latter hormone with and without pretreatment). In addition the effects of acute and chronic exposure to cold (5° C) were also examined because of the endocrine reactions presumably taking place under those conditions.

MATERIAL AND PROCEDURE

A stock of 115 female adult Wistar rats (230–340 gm.) was used. The animals were kept in individual cages and fed Ralston Purina laboratory chow for rats. Each rat acted as its own control. Two, 3 or even 4 experiments were sometimes performed on the same animal but they were staggered in such a way that at least one week elapsed between any two of them. This interval was considered adequate for the disappearance of any delayed hormonal effect.

Blood glucose and K were determined after an 18-hour fast unless otherwise specified. Tail blood samples were taken under light Nembutal anesthesia (3 mg./100 gm. on an average). Immediately after the first tail blood sample had been obtained, a dose of 0.5 gm. of glucose/Kg. body weight in 50% solution (Parke, Davis & Co.) was injected rapidly into the saphenous vein. Eight or nine other 0.1 ml. samples of blood were taken between 10 and 30 minutes after the injection, the exact timing being recorded to the nearest one-half minute. Blood glucose levels were determined by the Somogyi-Nelson micromethod (5). In one experiment (chronic cold exposure) the insulin sensitivity of the animals was tested by repeating the procedure half an hour after the first glucose tolerance test and immediately following the intravenous injection of 0.1 U./Kg. of crystaline insulin.

HORMONAL TREATMENTS. Doses, specific numbers and average weights of animals injected are given in the tables.

Epinephrine hydrochloride (*Winthrop*) was a synthetic preparation in 1% solution. After further dilution in saline, 20 or 100 μg. were injected intraperitoneally per animal.

Glucagon (crystalline Lilly CT-871 = 1 mg./ml., pH 2.5 with an insulin content of less than 5.10^{-2} U. per mg.) Doses of 10, 50 and 100 μg per rat (the first diluted in a phenol solution pH 2.5) were administered intraperitoneally.

Insulin (crystalline glucagon-free Lilly 40 U./ml.) Doses of 0.2 or 0.5 U. were given intraperitoneally, each dose being dissolved in 1 cc. of saline.

L-Thyroxine Na (Nutritional Biochemicals Corporation) was dissolved in a NaOH solution pH 8 to a final strength of 0.5 mg./ml. and stored in the cold room up to 3 days. In the acute experiment 50 or 100 μg. were injected intraperitoneally into each rat. In the chronic experiment the animals received 20 μg. intraperitoneally at 10 A.M. daily for 7 days and a second intraperitoneal injection of 50 μg. at 5 P.M. on the last day of treatment, the animals being then fasted overnight before the test. Body weight and food intake (ground chow) were recorded daily.

Hydrocortisone acetate (Cortef-Upjohn Company). The suspension was diluted after shaking in distilled water and a dose of 1 or 5 mg. injected intraperitoneally.

COLD EXPERIMENT. Cold exposure was carried out in a cold room at 5° C. In the acute experiment the animals were kept in individual metallic cages for 5 hours, without any protection. In the chronic experiment three rats were placed in the same cage with sawdust at the bottom of the cage. In both instances the animals were injected with Nembutal in the cold room and brought immediately to 20° C. They received the glucose load within the next 30 minutes.

RESULTS AND DISCUSSION

Evaluation of the Method. The advantages of this method are its rapidity, the fact that it does not involve isotopes and the easy statistical processing of the data. The physiological significance of these data however, requires critical evaluation. In the opinion of the authors, K, the velocity constant of the rate of disappearance of glucose from the blood after the intravenous load of glucose, can be considered to be representative of the rate of utilization of the endogenous liver output of glucose, provided 2 assumptions are correct: (1) that there is no more endogenous glucose output and (2) that there is no modification of the amount and/or effectiveness of insulin bound to the tissues after the i.v. load of exogenous glucose.

The Liver and K

The first assumption has been validated in normal dogs and human beings by experiments where catheter sampling at the hepatic and sometimes also at the portal levels were conducted after an intravenous glucose injection (6, 7, 8). The fact that the best fitting equation for the data is usually a pure exponential shows also (though only indirectly) that after the i.v. injection of glucose, the blood sugar tends toward a zero value. This would suggest that the liver no longer pours glucose into the hepatic veins during the hyperglycemic phase. Unfortunately it has not been directly proven that the various hormonal treatments used in this study do not interfere with the effect of exogenous glucose on the endogenous glucose outflow. Under these conditions it can only be assumed that the hypothesis is valid in those experiments where a straight line can be accurately drawn on a semilogarithmic scale. This is not the case when K is low (in diabetic or paradiabetic conditions) in which case small errors in the glucose determinations affect the accuracy of the best fitting mathematical representation of the data. In such cases, the apparent rate of glucose utilization could be altered and reduced by some continuous glucose output from the liver (see below). It is therefore safe to state that K represents the algebraic sum of a positive glucose uptake and a negative glucose output, and that in a majority of instances the latter factor is eliminated by the glucose load so that K represents only the first factor.

The Pancreas and K

It has been demonstrated that the value of K rises immediately when insulin is administered i.v. together with glucose (1, 2). The rate of decrease of the blood sugar levels observed when glucose alone is given can consequently be considered as representative of the rate of glucose uptake taking place before the test only if in addition to a complete cessation in the hepatic glucose outflow there is no modification in the amount and/or effectiveness of insulin bound to the tissues throughout the experimental procedure. The fact that K does not vary with the degree of hyperglycemia (1) and does not vary when the test is repeated (1, 2) has been taken as demonstrating that the transient hyperglycemia due to the test does not modify the rate of glucose uptake. This does not necessarily mean that there is no modification in the secretion of insulin by the β cells. It has indeed been shown recently that there is a sharp and transient increase in serum insulin-like activities in human beings after a rapid intravenous injection of glucose (9). This experiment opens new perspectives on the difficult problem of the relationship between insulin-like activities demonstrable in blood and tissues (10).

Epinephrine

It has been recently demonstrated that the epinephrine content of the rat plasma is approximately 7 μg./l. (11). It is therefore recognized that the intraperitoneal dose of 20 μg. per rat used in this study was somewhat unphysiological and that the dose of 100 μg. was definitely pharmacological. The clinical status of rats treated with 20 μg. remained excellent throughout the experimental period but after administration of 100 μg. the animals exhibited during the first 60 minutes marked apathy with some cyanosis and blanching of the extremities. From the data presented in Table 1 it is apparent that the response to epinephrine depended on the

TABLE 1. EFFECT OF *epinephrine* ON BLOOD SUGAR (G) AND GLUCOSE UTILIZATION (K) OF ADULT FEMALE RATS, FASTED FOR 18 HRS.

Dose (in micrograms /rat)	t	n	Average wt.	G_0	G_1	K_0	K_1
20 I P	1 hr.	8	266	70 ± 11	127 ± 8	1.6 ± 0.2	1.4 ± 0.4
	2 hrs.	7	288	69 ± 19	85 ± 19	1.5 ± 0.4	3.9 ± 1.6
100 I P	1 hr.	8	241	64 ± 14	148 ± 18	1.5 ± 0.4	−0.1 ± 0.6
	2 hrs.	6	219	73 ± 13	172 ± 18	1.7 ± 0.4	1.0 ± 0.8

Figures following ± are standard deviations.
t = time interval between the injection of epinephrine and the second glucose tolerance test
G_0 and G_1 = fasting blood sugars immediately before the first and second tolerance tests in mg/100 ml
K_0 and K_1 = rates of glucose utilization before and after epinephrine, expressed as percentage of blood glucose decrease per minute.

dose injected. After the intraperitoneal injection of 20 μg. of epinephrine per rat, the blood sugar G rose after one hour and there was a trend toward a return of G to normal values after 2 hours. A higher dose of epinephrine (100 μg./rat) led to a higher and more sustained hyperglycemic response. The hyperglycemia may be produced both by an increased hepatic glucose output and by a decreased permeability of peripheral tissues and it is difficult to define the relative importance of these two factors. With 20 μg. the liver probably played the determinant role as there was no decrease in the apparent rate of glucose utilization. How the liver is able to supply such an amount of glucose is explained by the finding that glycogen is simultaneously decreased in muscles and increased in liver (12) under epinephrine treatment in rats. It seems reasonable to assume that new molecules of glucose are constantly built up by the liver from lactic acid and/or other metabolites (13) resulting from muscle glycogenolysis and are in turn either delivered to the blood stream or polymerized to glycogen. With 100 μg. the hyperglycemia was accompanied by a severe decrease of K, a finding similar to that described in men by Amatuzio (14). The decrease of K was so evident that an impaired permeability of peripheral tissues seems involved. It has been hypothesized that a rapid hydrolysis of muscle glycogen could result in an increased intracellular hexose phosphate content and a non-competitive inhibition of glucokinase (15). The peripheral vasoconstriction observed here could also lead to an impairment of cellular metabolism and a secondary decrease in glucose utilization. The experimental evidence reported in the literature for inhibition of glucose uptake after epinephrine treatment is not fully convincing. While there is good agreement among investigators for an inhibition of the insulin effect on glucose uptake in eviscerated rats (16) and dogs (17) and in rat diaphragm *in vitro* (18), no direct effect of ephinephrine on the basal glucose uptake was seen in eviscerated preparations (16, 17) or the effect was doubtful in the case of rat diaphragm (18). In the present experiment, the decreased value of K after a large dose of epinephrine was also conditioned by the outflow of glucose from the liver which interfered with and continued during the test. This was obviously the case since positive K values were observed in a few instances, a phenomenon which could be only explained by the fact that in spite of the additional intravenous load of exogenous glucose, the liver was still pouring out endogenous glucose.

From the data in Table 1 it is also obvious that the time factor is important and that K tended to come back to normal with 100 μg. or even went above normal with 20 μg. following a period of 2 hours. It is probable that this secondary enhancement of K is due to the stimulation of β cells by the hyperglycemia. It has indeed been demonstrated that in rats injected with epinephrine, the glycogen stores of muscles are rebuilt only in the presence of a normal pancreas (12). This strong reaction of β cells

must be one of the mechanisms explaining why it has never been possible to observe a permanent diabetes after a chronic treatment with epinephrine (19).

Glucagon

The doses of glucagon used in this study were large. Table 2 gives the results obtained with a single intraperitoneal injection of 10, 50, or 100 μg. In fasted animals, variations in blood glucose (G) half an hour to 18 hours after treatment were not consistent. The only significant modification was

TABLE 2. EFFECT OF *glucagon* ON BLOOD SUGAR (G) AND GLUCOSE UTILIZATION (K) OF ADULT FEMALE RATS, FASTED FOR 18 HRS. OR FED AD LIBITUM

Dose (in micrograms/rat)	Dietary status	t	n	Average wt. (gm.)	G_0	G_1	K_0	K_1
10 I P	Fasted	2 hrs.	11	228	74±11	75±12	1.6±0.3	1.8±0.8
50 I P	Fasted	30 min.	12	289	67±12	82±12**	1.8±0.6	2.7±1.0*
	Fasted	90 min.	12	229	62±11	82±11***	1.7±0.4	2.3±0.7**
100 I P	Fasted	30 min.	8	262	73±11	66±12	1.7±0.5	1.8±0.7
		90 min.	8	265	71±11	72±10	1.7±0.5	2.4±0.5
		18 hr.	5	222	73±12	62±11	1.6±0.4	1.8±0.6
100 I P	Fed	1 hr.	5	271	89±10	121± 5***	2.1±0.9	2.5±0.6

* $P<0.05$.
** $P<0.02$.
*** $P<0.01$.
Figures following ± are standard deviations.
t = time interval between the injection of glucagon and the second glucose tolerance test
G_0 and G_1 = blood sugars immediately before the first and second tolerance tests in mg./100 ml.
K_0 and K_1 = rates of glucose utilization before and after glucagon, expressed as percentage of blood glucose decrease per minute.

a small rise of G at 30 and 90 min. following injection of a 50 μg. dose. On the other hand, the blood sugar levels of the group of rats fed *ad libitum* and injected with 100 μg. of glucagon showed a more obvious rise one hour after injection.

That variations in blood glucose were of small magnitude in fasted animals and only a little more conspicuous in fed animals was probably related to the fact that the glucagon used in this study was administered intraperitoneally. A greater rise of blood glucose has been reported after intravenous administration (20). The moderation of the present effects, when compared to those observed with epinephrine can probably also be explained in the light of the reported lack of influence of glucagon on muscle phosphorylase (21). If the muscles offer no additional material of glycolytic origin to the liver, the source of hepatic glucose output is restricted and this could limit the extent of hyperglycemia. Therefore even a marked liver glycogen mobilization can perhaps occur without marked

hyperglycemia (20) and with only a small increase in peripheral glucose disposal. This was apparently the case since the rates of glucose utilization showed no change except in fasted rats treated with 50 μg. of glucagon in which a significant increase was observed. The conclusion appears warranted that glucagon at least does not impair glucose utilization. In the single case where glucose utilization was enhanced this effect can conceivably be related to an insulin hypersecretion resulting from hyperglycemia or to a direct effect of glucagon on peripheral tissues. However, the interpretation of this enhancing effect on glucose utilization is debat-

TABLE 3. EFFECT OF *crystalline insulin* ON BLOOD SUGAR (G) AND GLUCOSE UTILIZATION (K) OF ADULT FEMALE RATS, FASTED 18 HRS.

Dose (in units/rat)	t	n	Average wt. (gm)	G_0	G_1	K_0	K_1
0.5 I P	1 hr.	5	231	65 ± 8	2 ± 4	1.4 ± 0.2	11.4 ± 2.3
0.2 I P	1 hr.	5	238	56 ± 14	12 ± 14	1.8 ± 0.6	4.9 ± 1.9
	2 hrs.	6	246	65 ± 13	7 ± 9	2.0 ± 0.5	0.7 ± 1.5
	5 hrs.	7	228	64 ± 11	65 ± 10	1.7 ± 0.3	0.1 ± 0.6
	18 hrs.	6	233	72 ± 12	68 ± 12	1.7 ± 0.4	2.0 ± 1.3

Figures following ± are standard deviations.
t = time interval between the injection of insulin and the second glucose tolerance test
G_0 and G_1 = fasting blood sugars immediately before the first and second tolerance tests in mg./100 ml
K_0 and K_1 = rates of glucose utilization before and after insulin, expressed as percentage of blood glucose decrease per minute.

able because of its inconstancy, of the large dose utilized and since even a small contamination of glucagon with insulin may be a source of misunderstanding (22).

Insulin

One hour after the intraperitoneal injection of 0.5 U. of insulin the fasting blood glucose dropped to near zero value (Table 3). At the same time, the rate of glucose utilization K was extremely high. An attempt to test another lot of rats 90 minutes after the injection of the same amount of insulin did not succeed as the animals were in shock. With a smaller dose of insulin (0.2 U. i.p.) the fasted rats were in a much better condition. Again a sharp drop in blood sugar and a marked rise of K were observed one hour after the injection. However, K decreased after 2 hours, the blood glucose remaining at a very low level, and five hours after insulin injection the average rate K approached zero while the blood sugar had returned to normal levels. In fact, as in the case of epinephrine, positive values for K were sometimes found.

That hypoglycemia due to insulin is related mostly to an increase in the rate of glucose utilization by non-hepatic tissue has been recognized

for a long time. When insulin is injected intravenously with glucose a nearly immediate effect on K is observed in man, dogs and rats (1, 2), this increase being proportional to the logarithm of the dose of hormone (2). In the present experiment, insulin was injected intraperitoneally under fasted conditions. In that way emphasis was not put on these well known primary effects of insulin but on the efficiency of the animals' own regulatory mechanisms. The delayed decrease in glucose tolerance can be safely ascribed to a secondary reaction of the medullo-adrenals. It has been demonstrated that the hypoglycemia acts on the hypothalamus which in turn stimulates the adrenal medulla (23). It is therefore not surprising that K tended toward values as low as those observed after the injection of a high dose of epinephrine. As seen when discussing the effects of epinephrine treatment it is probable that the return of the blood sugar to its original level following the administration of insulin was due to a secondary increased output of glucose by the liver and to a decrease in the rate of glucose uptake.

L-*Thyroxine*

The daily secretion of thyroid hormones by normal rat has been evaluated to be equivalent to 5 μg. of thyroxine (24). The doses of 20, 50 and 100 μg. utilized in the present study are therefore relatively high. Two hours after a single intraperitoneal injection of thyroxine (Table 4) a significant rise in the rate of glucose utilization was observed. This rise increased in magnitude after 18 hours but faded after 24 hours. A small but significant hyperglycemia was also observed after 18 hours and persisted

TABLE 4. EFFECT OF L-*Thyroxine* ON BLOOD SUGAR (G) AND GLUCOSE UTILIZATION (K) OF ADULT FEMALE RATS, FASTED 18 HOURS

Dose (in micrograms/rat)	t	n	Average wt. (gm.)	G_0	G_1	K_0	K_1
100 intraperitoneal	2 hrs.	19	239	64 ± 14	68 ± 10	1.8 ± 0.6	2.6 ± 0.7***
50 intraperitoneal	2 hrs.	15	288	66 ± 12	61 ± 14	1.7 ± 0.5	2.5 ± 0.9***
	18 hrs.	10	284	61 ± 15	77 ± 5**	1.7 ± 0.7	3.6 ± 1.0***
	24 hrs.	7	247	60 ± 12	74 ± 9*	1.9 ± 0.6	2.1 ± 3.9
	18 hrs.†	9	274	61 ± 15	75 ± 6***	1.7 ± 0.7	2.7 ± 0.9

Figures following ± are standard deviations.
* $P < 0.05$.
** $P < 0.02$.
*** $P < 0.01$.
t = time interval between the injection of 1- thyroxin and the second glucose tolerance test
G_0 and G_1 = fasting blood sugars immediately before the first and second tolerance tests in mg/100 ml
K_0 and K_1 = rates of glucose utilization before and after 1-thyroxin, expressed as percentage of blood glucose decrease per minute.
† In this group the animals received 20 micrograms at 10 A.M. daily for 7 days and a second injection of 50 micrograms at 5 P.M. on the last day of treatment, the animals being then fasted for 18 hours before the test.

after 24 hours. The striking observation of a rapid increase in K was a little surprising. However, other and even more rapid effects of thyroxine *in vivo* have been described. Marked decrease of the P:O ratio of heart mitochondria can be noted as fast as 10 minutes after injection of a very high dose of thyroxine (25). The present increased glucose utilization is in accordance with former experiments demonstrating *in vitro* an increased glucose uptake by the rat diaphragm (26) as well as an increased glucokinase activity in muscle (27). This increase in K, associated with a small but definite increase of the blood sugar level after a single injection of thyroxine leads to the conclusion that there was a greater turnover rate of glucose in fasted rats. The corollary is that there was also an increased liver glucose output, an assumption in accordance with the previous finding of an increased liver glucose-6-pase activity (28).

During the chronic experiment (20 μg./day for 7 days) the appetite of the animals was unmodified (16 gm. of ground chow daily). While no diarrhea was observed, the weight decreased slightly (an average of 10 gm.) and some irritability occurred. The rise in K noted 17 hours after the last injection of 50 μg. of thyroxine was not significant. On the other hand, a small degree of hyperglycemia was observed. The fact that K was, if not significantly higher than normal, at least unimpaired, leads to the conclusion that this small hyperglycemia was related to an increased glucose output and not to a decreased glucose tolerance. In relation to this, no atrophy of the islets of Langerhans has been reported in chronically treated rats, in fact a relative increase of their weight has been observed (29).

In hyperthyroid patients increase (14) and decrease (30) in the rate of glucose utilization have been reported but these apparently contradictory results are probably related to the nutritional status of the investigated patients and the stage of their disease.

Hydrocortisone

Two hours and 18 hours after the injection of 1 mg. of hydrocortisone (Table 5) blood sugar levels and rates of glucose utilization were unchanged. Two hours after the injection of an even larger dose of hydrocortisone (5 mg. intraperitoneally) the average fasting glucose level showed no change but there was a small, significant decrease in in K. In human beings (31) and mice (32) a decrease in the rate of glucose disposal has been observed after acute treatment with corticoids. This apparent decrease in glucose utilization does not seem to be related to a decrease in peripheral glucose uptake as no such decrease was found in eviscerated rats (33) or in rat diaphragms *in vitro* (review by Renold, 34).

On the other hand, the liver is known to react rapidly to corticoids. Two hours after *in vivo* cortisone treatment liver slices of alloxan-adrenalectomized rats already exhibit an increased glucose output (35). This output can display *in vivo* a 7-fold rise (36) and one wonders therefore if the

TABLE 5. EFFECT OF *hydrocortisone acetate* ON BLOOD SUGAR (G) AND GLUCOSE UTILIZATION (K) OF ADULT FEMALE RATS FASTED FOR 18 HRS.

Dose (in mg./rat)	t	n	Average wt. (gm.)	G_0	G_1	K_0	K_1
1 (I P)	2 hrs.	9	227	59 ± 8	66 ± 9	1.8 ± 0.6	2.1 ± 0.8
	18 hrs.	8	238	61 ± 9	65 ± 9	2.2 ± 0.3	2.7 ± 0.5
5 (I P)	2 hrs.	13	235	69 ± 11	72 ± 11	1.8 ± 0.4	1.4 ± 0.7*

Figures following ± are standard deviations.
* $P < 0.05$.
t = time interval between the injection of hydrocortisone acetate and the second glucose tolerance test
G_0 and G_1 = fasting blood sugars immediately before the first and second tolerance tests in mg/100 ml.
K_0 and K_1 = rates of glucose utilization before and after hydrocortisone acetate, expressed as percentage of blood glucose decrease per minute.

small apparent decrease in K observed here with a very high dose of cortisone was not again due to some continuous glucose output from the liver proceeding in spite of the hyperglycemia induced by the test. Furthermore, it has been shown that a protracted treatment with high doses of cortisone resulted secondarily in an opposite effect, i.e. in a significant rise in glucose utilization in rat (3), man (37) and guinea pig (38). It is possible that in these experiments the increased hepatic glucose output has resulted in a secondary stimulation of the β cells which could in turn account both for this increase in glucose utilization and the hypertrophy of the islets of Langerhans (39).

Cold Exposure

Acute Experiment. Four hours following exposure to 5° C, the animals exhibited piloerection and cyanosis of the extremities, but these signs were less pronounced than those observed under severe hypoglycemia. The blood sugar was unchanged and a small but significant decrease in K was found (Table 6). Two explanations could be offered to account for this small decrease in K. The first would be epinephrine release. The second would be a rapid depletion of carbohydrate stores resulting from increased energy expenditure under fasting conditions. This rapid exhaustion could lead to a lowering of the extent of glucose utilization. The sharp depletion of liver glycogen described by Masoro (40) is probably part of this picture.

Chronic Experiment. After a 14-day exposure to cold, the animals seemed unaffected. There were no trophic lesions of the ears, paws, and tails. Some vasodilation and hyperemia were noticed when puncturing the tail veins. There was again no change in blood glucose levels but, at variance with the first experiment, there was a small but significant increase in K. Thyroid hyperactivity and increased food intake are probably involved, as well as an increased insulative capacity of the pelage (41). Another

TABLE 6. EFFECT OF *cold exposure* (5° C) ON BLOOD SUGAR (G), GLUCOSE UTILIZATION (K) AND INSULIN SENSITIVITY (K_2) ON ADULT FEMALE RATS FASTED FOR 18 HRS.

Duration of cold exposure	n	Average wt. (gm.)	G_0	G_1	K_0	K_1	K_2
4 hrs.	17	213	62 ± 9	58 ± 10	1.7 ± 0.3	1.2 ± 0.4***	—
14 days	10	259	67 ± 12	66 ± 12	1.5 ± 0.2	2.0 ± 0.7*	8.4 ± 2.9
0 (control group)	5	233	64 ± 7		1.5 ± 0.4		4.5 ± 0.8

Figures following ± are standard deviations.
* $P < 0.05$.
*** $P < 0.01$.
G_0 and G_1 = fasting blood sugars immediately before the first and second tolerance tests in mg./100 ml.
K_0 and K_1 = rates of glucose utilization before and after cold exposure.
K_2 = rate of glucose utilization after a combined glucose-insulin intravenous load (see text).

evidence for alteration in carbohydrate metabolism is given by the striking increase in insulin sensitivity reported in Table 6. It is readily seen from the data that, whatever index of insulin sensitivity is considered (the absolute value of K_2, the difference in the response $K_2 - K$ or the ratio $K_2 : K_1$) the standard dose of 0.1 U./Kg. produced a higher response in "cold-adapted" rats than it did in a control group of rats tested at the same time. Similar results have been obtained by Baker (42) who has also shown a decreased insulin content of the pancreas (43).

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PRODUCTION OF OVULATION IN THE HYPOPHYSECTOMIZED RHESUS MONKEY[1]

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ABSTRACT

The experimental induction of ovulation in the hypophysectomized rhesus monkey is reported. This was produced by appropriate treatment with desiccated thyroid, porcine FSH and HCG during a time interval roughly equivalent to the follicular phase of the menstrual cycle.

WHILE the hypophyseal regulation of ovarian function in rodents and certain other species has been rather clearly delineated, the nature of the hormonal control of ovarian activity in primates remains obscure. Attempts to reproduce experimentally the sequential ovarian changes observed during the normal menstrual cycle have, in large measure, been unfruitful (see 1). Two possible reasons for some of the difficulties encountered in such experiments are that they were performed in animals possessing intact pituitary glands, and that gonadotropins of infra-primate origin were generally used. The latter consideration became important with the discovery that growth hormone preparations obtained from monkey pituitary glands were physiologically effective, while those derived from glands of non-primate forms were ineffective in the rhesus monkey (2, 3). Recently, van Wagenen and Simpson (1, 4, 5) have made the important observation that the treatment of immature and adult female rhesus monkeys with gonadotropin preparations of primate origin resulted in multiple ovulation with a frequency never before attained. These experiments, however, were performed in intact animals, and the possibility remained that endogenous gonadotropin secretion played a role in the ovulatory response which they observed.

MATERIALS AND METHODS

Thirteen hypophysectomized female rhesus monkeys (*Macaca mulatta*) were used in this study. They were hypophysectomized by the parapharyngeal route (6) and were maintained on a natural diet supplemented with a 10% glucose solution as a drinking fluid. Tap water was also provided. They were individually caged in air-conditioned quarters held at 78° F.

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[2] National Research Fellow in the Medical Sciences.

The hormone preparations used were as follows: a) Saline homogenates of monkey anterior pituitaries; b) Follicle Stimulating Hormone—Porcine (Armour lot no. R377201); c) Luteinizing Hormone—Equine (Armour lot no. R377279); d) Human Chorionic Gonadotropin (Parke, Davis and Co., Antuitrin S).

Exploratory laparotomies were performed under Nembutal anesthesia at varying intervals following the initiation of treatment and the ovaries, fallopian tubes and uterus were examined. When appropriate, the ovaries were removed, fixed in Bouin's fluid, serially sectioned at 10 μ, and stained with hematoxylin and eosin. As many as five laparotomies were performed in some animals without ill effects. Supportive adrenalcortical therapy was not given.

Completeness of hypophysectomy was confirmed by the cessation of growth, blanching of the sex skin, atrophy of the ovaries and accessory organs of reproduction, increased insulin sensitivity and increased glucose tolerance. Six of the animals used in this study are still alive. The remainder succumbed to severe hypoglycemia. Examination of the sella turcica of these animals failed to reveal any pituitary remnants.

RESULTS AND DISCUSSION

In the initial phase of this study the ovarian response to a saline homogenate of monkey pituitaries was tested in several long-term hypophysectomized juvenile or adult female monkeys. One ovary was removed for control purposes just prior to the beginning of treatment. The animals were given daily intramuscular injections of homogenate equivalent to 5–20 pituitary glands. Changes in the sex skin were noted usually within the first week and occasional laparotomies confirmed ovarian enlargement and the presence of many large translucent graafian follicles. Illustrative are the responses obtained in two animals:

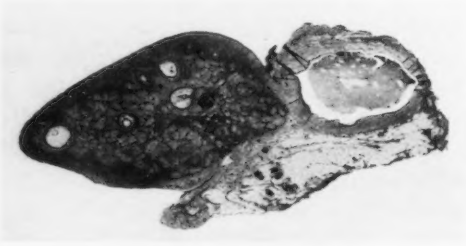

FIG. 1A. Right ovary of young mature female (no. 96) one year after hypophysectomy. ×6.

Monkey no. 96 was hypophysectomized 12 months before the initiation of treatment. The control ovary (Fig. 1A) was grossly atrophied and contained a small number of undeveloped follicles, a few of which were in the early antrum stage. Beginning on the fifth day of treatment, the sex skin reddened and became edematous, and as treatment was continued the redness and edema became progressively more pronounced. On the 24th day, the left ovary, which was considerably enlarged, was excised and found to contain large follicles with a thin membrana granulosa. No evi-

dence of luteinization or ovulation was observed (Fig. 1B). Monkey no. 106 hypophysectomized for two months was treated in identical fashion and responded in a similar manner. In this animal, however, treatment was continued for 43 days and the interesting observation was made that despite continued injections of the monkey pituitary preparation, refractoriness appeared to occur as evidenced by a regression in the size of the ovary and uterus and an atrophy of the sex skin. Examination of the remaining ovary at this time revealed rather large cystic follicles without luteinization.

Similar results (follicular development, production of cystic ovaries, increased estrogen secretion as evidenced by uterine growth and sex skin development, and occasional refractoriness) were obtained when FSH (5–10 U./day) and HCG (500–2500 U./day) were substituted for the pituitary homogenates. In these experiments, the FSH was given for 5 days followed by combined treatment with FSH and HCG. A representative

FIG. 1B. Left ovary of same animal (no. 96) following 24 days of daily intramuscular injections of a saline homogenate of fresh-frozen monkey pituitary glands (each daily dose equivalent to 5 whole monkey pituitaries). ×6

ovarian response to such treatment is illustrated in Figure 2. While limited areas of luteinization were observed on occasion (Fig. 3), and hemorrhagic follicles were frequently encountered, ovulation in response to such treatment, with one exception, was not induced. This was the case whether the hormones were administered in single or divided doses, given subcutaneously, intramuscularly or intravenously, injected as aqueous solution or in gelatin vehicle, given as single or combined injections, or when equine LH was substituted for the chorionic hormone.

The one exception (no. 127) was treated with 10 U. FSH per day for 14 days at which time the dose of FSH was reduced to 5 U. and 2500 U. of HCG per day was instituted. The latter was given in two daily doses each equally divided in one subcutaneous and one intravenous injection. On the 21st day, the left ovary (5×8×15 mm.) was removed and examined. It contained several large hemorrhagic follicles most of which exhibited luteinization of the granulosa cells (Fig. 4). The largest of these possessed

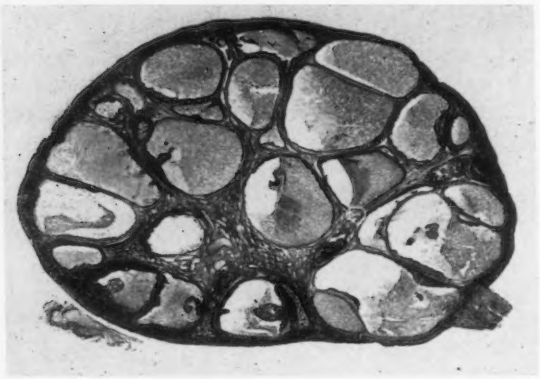

FIG. 2. Left ovary of immature female (no. 126) hypophysectomized one month before initiation of experiment. Treated for 5 days with 5 U. FSH followed by 13 days of combined injections of 5 U. FSH and 500–2000 U. HCG. Laparotomy and excision of right ovary revealed cystic and hemorrhagic follicles but no ovulation. Dose of FSH reduced to 1 U./day and 50–100 U. equine L.H./day substituted for HCG. This treatment was continued for 8 days and the left ovary removed. ×5.

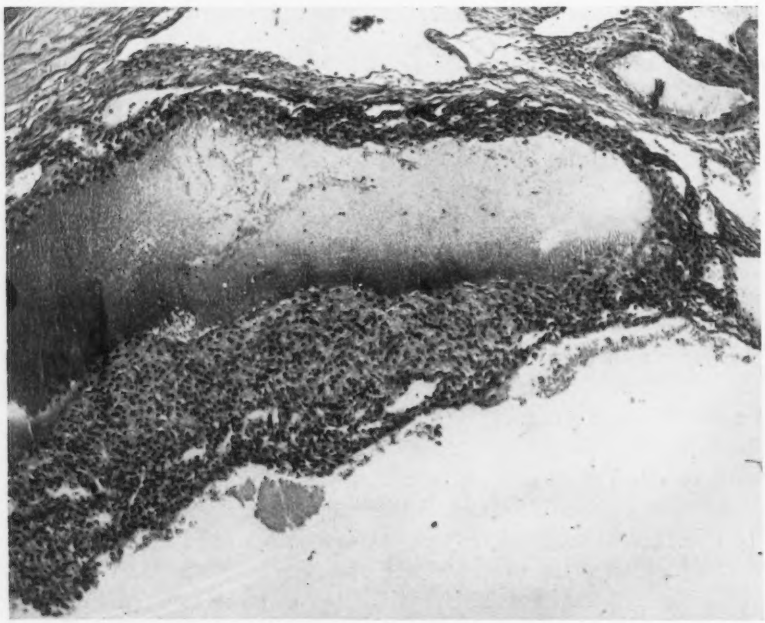

FIG. 3. Follicle of gonadotropin treated hypophysectomized monkey showing lutein plaque. ×100. This animal, an immature female (no. 121) was hypophysectomized 2 months before the experiment. Treatment: 5 days with 10 U. FSH/day; 5 days with 20 U. FSH/day; 6 days with decreasing doses of FSH to 1 U./day combined with 2000 U. HCG/day divided into equal intravenous and subcutaneous or intramuscular doses.

FIG. 4. Probable ovulation in hypophysectomized monkey (no. 127). ×5.

Hypophysectomized one month before start of treatment. 10 U. FSH/day for 12 days, 5 U. FSH plus 1000 U. HCG/day for 9 days.

an ovulation point and is thought to have ovulated inasmuch as examination of serial sections of this follicle failed to reveal an ovum. Extensive luteinization was evident in an adjacent follicle (Fig. 5) but a rupture point was not present.

The inability to induce ovulation in these experiments and the invariable presence of cystic follicles suggested excessive stimulation by FSH. In the next series of three animals, the dose of FSH was accordingly reduced to 1 U. given subcutaneously twice daily for 6 days. On the 7th day, this dose was reduced by a factor of 10 and supplemented with 500 U. HCG given twice daily. Laparotomy was performed on the 14th day of treatment. The monkeys used in this series were adult females at the time of hypo-

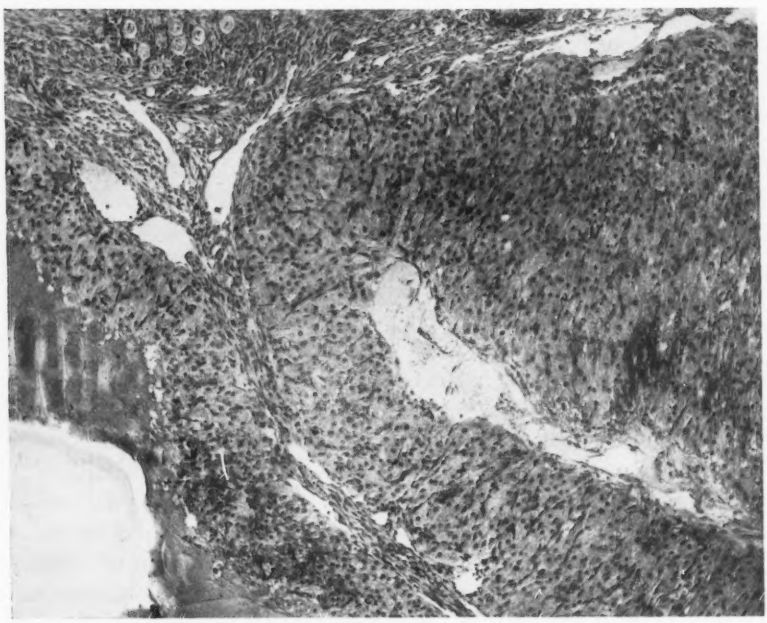

FIG. 5. Same ovary (no. 127) showing complete luteinization of a follicle. ×100.

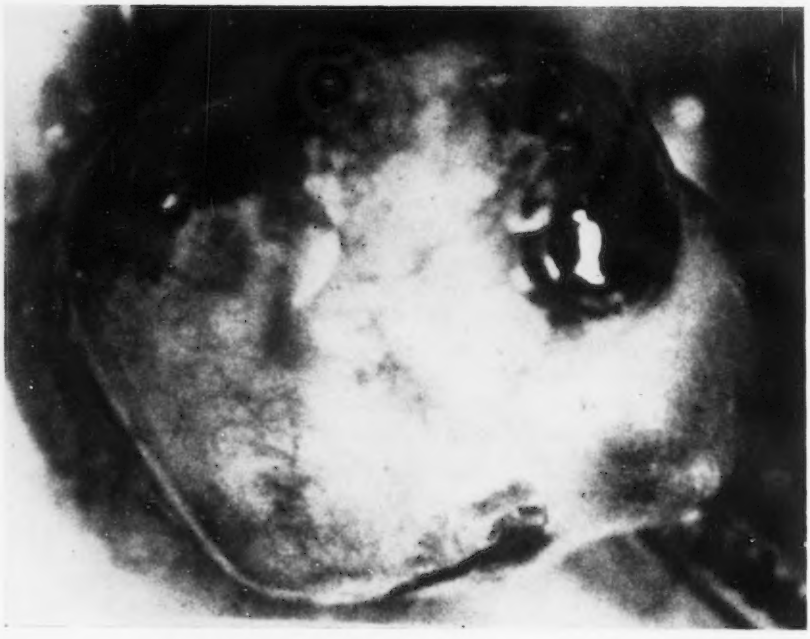

FIG. 6. Ovulating ovary of monkey no. 138 photographed *in situ* at laparotomy. See text for details of experiment.

physectomy two months previously, and were given $\frac{1}{8}$ grain of desiccated thyroid orally per day beginning one week before the start of gonadotropin treatment. In one animal (no. 138) multiple ovulations were produced in one ovary, the stigmata being clearly evident and oozing follicular fluid at the time of laparotomy (Fig. 6). Sections of this ovary, which was the largest ever observed by us, confirmed recent ovulation and incipient luteinization (Fig. 7). The other ovary of this monkey was closely enveloped by the fimbria and showed luteinization but not ovulation. Whether this complete and adhesive encapsulation of the ovary by the fimbria is a pathological or physiological phenomenon is unknown at present.

Ovulation was not produced in the remaining two animals of this series, although these were treated in a manner identical to the above. Their ovaries, however, were strikingly hypertrophied and contained a large number of luteinized follicles in which entrapped ova were found.

Several salient points are brought out by this preliminary study: a) The atrophic ovaries of monkeys hypophysectomized for as long as one year respond as readily to the injection of FSH as those of animals hypophysectomized for a short period of time; b) Follicle stimulation is readily accomplished with FSH preparations of non-primate origin; c) Continued

FIG. 7. Section of same ovary (no. 138). ×5. See text for details.

follicular stimulation with homologous or heterologous FSH preparations eventually results in refractoriness and a regression in ovarian size and estrogen secretion; d) Excessive follicle stimulation achieved too rapidly obviates ovulation in response to subsequent ovulating doses of chorionic gonadotropin; e) The evidence suggests that gradual stimulation of the follicles with small doses of FSH followed by large doses of HCG in a time span corresponding to the follicular phase of the menstrual cycle is most likely to result in ovulation; f) The continued study of the experimental induction of ovulation in the hypophysectomized rhesus monkey should provide fundamental information toward the elucidation of the physiological regulation of ovarian function in primates.

Acknowledgments

The authors are indebted to Miss Anna Morse and Mr. John F. Cimerol for technical assistance.

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POLYARTHRITIS PRODUCED IN RATS BY TREATMENT WITH PROLACTIN AND GROWTH HORMONE

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ABSTRACT

Prolactin and growth hormone when given in combination cause polyarthritis in the adrenalectomized desoxycorticosterone-maintained (1 mg. every second day) and in the hypophysectomized rat. The pituitary hormones were administered separately in two daily subcutaneous injections of 0.5 mg. during eight days in the former animals, and in single daily injections of 1 mg. during 20 days in the latter. The dosage was then doubled until completion of the experiments, which lasted 15 and 30 days respectively.

In adrenalectomized rats, the joint lesions occurred suddenly after ten days of treatment and were acute in nature. Coincident to the development of arthritis, the animals showed hyperthermia and often died with hypoglycemic convulsions. In hypophysectomized rats, the reaction appeared more insidiously after 20 days' treatment and evolved in a more chronic manner.

Prolactin seems to play a greater role than does the growth hormone in the pathogenesis of these arthropathies, since their incidence is not related to the anabolic condition of the animals. Furthermore, it is suggested that the state of adrenal insufficiency may be an important predisposing factor.

POLYARTICULAR lesions have been reported in rats, following prolonged treatment with desoxycorticosterone (1, 2), lyophilized anterior pituitary extract (3, 4), and growth hormone (5, 6). However, these so-called hormonal arthritides develop only occasionally and the experimental conditions required for their production have not yet been clearly established. It was observed that the susceptibility of animals to arthritis is markedly increased after adrenalectomy, either alone (1, 2, 7, 8) or associated with gonadectomy (6). Hypophysectomized rats are likewise more prone to develop joint lesions when treated with growth hormone alone (9) or in combination with desoxycorticosterone (5).

The present report deals with polyarticular lesions that result in rats treated with prolactin (LTH) and growth hormone (STH). In order to provide the most favorable experimental conditions for the development of arthritis, the hypophyseal hormones were administered to both the hypophysectomized and the adrenalectomized rats; the latter received maintenance doses of desoxycorticosterone.

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METHODS

Seventy rats of the Sprague-Dawley strain (Holtzman Farm) were used; their body weights ranged from 110 to 130 gm. The animals were kept on Purina Fox Chow and tap water *ad libitum* throughout the experiments. The glandular extirpations were performed under ether anesthesia, using the lumbar approach for adrenalectomy and the parapharyngeal route for hypophysectomy. Adrenalectomized animals were given a maintenance dose of 1 mg. of desoxycorticosterone acetate (DCA), in the form of microcrystals suspended in 0.2 ml. of physiologic saline, subcutaneously every two days. Hypophysectomized rats were not used until 15 days after the operation, by which time the signs of hypopituitarism are well established.

The first experiment was carried out on 40 adrenalectomized female rats divided into four groups. Group I served as controls; Group II received LTH;[3] Group III, STH;[3] Group IV, the combined treatment. Each hormone was administered subcutaneously in a volume of 0.2 ml. of physiologic saline, in two daily doses of 0.5 mg. each for the first eight days; the dose then was increased to 1 mg. twice daily and was given at this level until the end of the experiment, which lasted 15 days.

The second experiment was composed of 30 hypophysectomized male rats also divided into four groups. The treatment followed the same pattern as in the previous experiment except that each pituitary hormone was given once daily, at the dose of 1 mg. for the first 20 days and 2 mg. for the last 10 days of the experiment.

Severity of the lesions was appraised according to an arbitrary scale of 0 to +++. The means of the readings made during the last three days are given in the Tables. At the end of each experiment, the rats were killed with chloroform, their organs were removed and fixed in Susa solution for subsequent weighing and histologic examination. The joints were stained with Masson's HPS trichrome and Periodic-Acid-Schiff techniques.

RESULTS

Experiment I: Polyarthritis in adrenalectomized rats:

The first joint lesions appeared on the tenth day, among the rats receiving both LTH and STH (Group IV). In the following three days, almost every animal of this group was affected. The acute inflammatory changes, consisting in hyperemia and swelling of the carpal and metatarsal regions, often appeared in the four paws simultaneously. These acute manifestations usually regressed and were followed by recurrent proliferative lesions confined to either one or two articulations (Fig. 1B). Coincident to the development of arthritis, some rats became prostrated and eventually died with convulsions (blood glucose, 40 to 60 mg. %). Sudden lowering of glycemia is a common feature in adrenalectomized rats exposed to any deleterious condition.

The earliest changes, observed upon microscopic examination of the paws, consisted in a diffuse edema of the subcutaneous tissue with very few inflammatory cells. At that stage, the reaction resembles the anaphy-

[3] The growth hormone (NIH-BGH-1) is a highly purified bovine preparation, and the prolactin (NIH-SP-1) is a highly purified sheep preparation estimated to contain 20 I.U./mg., both being distributed by the Endocrinology Study Section, National Institutes of Health, Bethesda, Md.

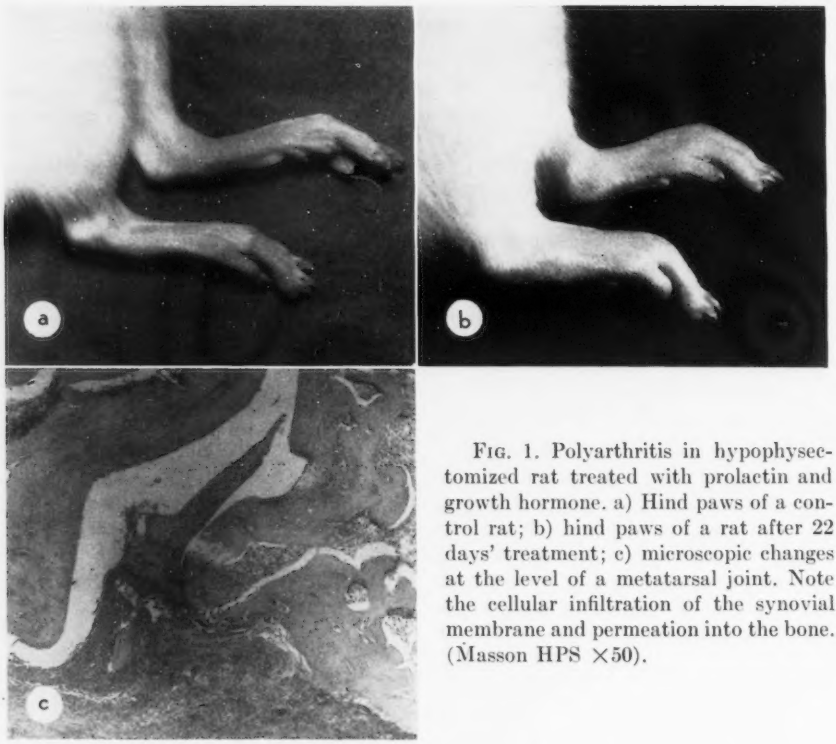

FIG. 1. Polyarthritis in hypophysectomized rat treated with prolactin and growth hormone. a) Hind paws of a control rat; b) hind paws of a rat after 22 days' treatment; c) microscopic changes at the level of a metatarsal joint. Note the cellular infiltration of the synovial membrane and permeation into the bone. (Masson HPS ×50).

lactoid edema produced in rats with dextran or other histamine releasers (8). The exudative process, however, soon evolves beyond that stage, extends to the surrounding tissue and gives rise to a diffuse cellulitis. The inflammatory cells consist mainly in mononuclear phagocytes, which infiltrate muscle fibers and tendon sheaths. Polymorphonuclear leukocytes are seldom seen; whenever present, they are localized in the inflamed bursae. As the inflammatory process reaches the articulation, there is an intense fibroblastic proliferation in the subcutaneous tissue. The synovial membrane is heavily infiltrated and extends into the articular cavity. In more severe lesions, some degree of osteolysis may be evidenced but in no instance was the articular cartilage affected (Fig. 1C).

The progression of the lesions was followed over a period of six days and their severity increased with the duration of the treatment. We do not know if complete ankylosis would eventually develop, but already after six days the animals moved with difficulty. Comparable lesions have also been noted in a few rats treated with LTH only (Group II); the lesions, however, were less proliferative. In the other groups, there was no evidence of arthropathies.

TABLE 1. EFFECT OF PROLACTIN AND GROWTH HORMONE IN ADRENALECTOMIZED RATS RECEIVING DESOXYCORTICOSTERONE AS SUBSTITUTION THERAPY

Groups	Treatment	No. rats	B.W. gain* (gm.)	Thymus (mg.)	Articular Lesions		Mortality
					Incidence	Severity†	
I	—	10	+51±2.86‡	475±17.3	0	0	0
II	LTH	10	+52±2.67	387±14.8	2	+	0
III	STH	10	+82±1.91	646±37.0	0	0	0
IV	LTH+STH	10	+59±2.37	498±24.4	9	++	4

* Mean initial body weight: 120 gm.
† Scale: 0 to +++.
‡ Mean ± Standard Error.

Experiment II: Polyarthritis in hypophysectomized rats:

In this series, arthritis appeared only after three weeks' combined treatment with LTH and STH. However, we were able to see, in subsequent experiments not reported here, that LTH given at a same dose level but in two daily injections was more efficient in producing arthritis than was a single treatment. In the present series, the articular inflammatory reaction developed more insidiously; it mostly involved the hind paws and tended to be confined to either the ankle or the tarsal region. Compared with those described above, the lesions were less proliferative and evolved in a more chronic manner, but their histologic aspect was essentially the same.

Perusal of the Tables shows that the body weight gain as well as the increase in thymus weight, normally resulting from STH treatment in hypophysectomized or adrenalectomized rats, was slightly reduced by the concurrent treatment with LTH. It will also be noted that the weight of adrenals in hypophysectomized animals was not significantly modified by treatment with either pituitary hormone. We may add that the ovaries of the LTH-treated rats were found to be completely atrophic at autopsy.

TABLE 2. EFFECT OF PROLACTIN AND GROWTH HORMONE TREATMENT IN HYPOPHYSECTOMIZED RATS

Groups	Treatment	No. rats	B.W. gain* (gm.)	Thymus (mg.)	Adrenals (mg.)	Articular Lesions		Mortality
						Incidence	Severity†	
I	—	6	+ 6±1.26‡	133± 6.6	10.3±0.16	0	0	0
II	LTH	6	+ 18±3.82	193±28.7	9.5±0.99	0	0	1
III	STH	8	+121±4.52	450±18.4	15.1±0.72	0	0	0
IV	LTH+STH	10	+108±1.73	399±17.9	12.4±0.64	10	++	0

* Mean initial body weight: 120 gm.
† Scale: 0 to +++.
‡ Mean ± Standard Error.

DISCUSSION

The above described experiments reveal that it is possible to produce polyarthritis in rats, regularly and in a rather short period of time, by the conjoint administration of two pituitary hormones, STH and LTH. Yet it is difficult to explain the pathogenic action of prolactin. As seen in the Tables, this hormone has no demonstrable anabolic effect that could account for its synergism with STH. It is already known that growth hormone can induce articular changes, but, as previously mentioned, only after prolonged intense treatment (5, 6, 9). In the present series, STH alone did not produce any articular deformities, presumably because of the short duration of treatment. Obviously, the inflammatory reaction that is produced in the joints within two or three weeks by treatment with STH and LTH has a distinct etiology from that occurring after several months of treatment with growth hormone. Besides, the arthropathies observed in our experiments evolved differently, with acute episodes, more in the pattern of a rheumatic disease.

Another factor that seems particularly relevant to the pathogenesis of such arthritis is the influence of adrenalectomy and hypophysectomy. Apparently, a complete or relative state of adrenal insufficiency plays a definite role in the development of the lesions; in all probability, this is attributable to a lack of glucocorticoid hormones (6). It should be mentioned that similar lesions have been noted in a few uninephrectomized rats receiving LTH plus DCA (11). Whether or not the DCA treatment is involved in the pathogenic process needs to be further verified. More likely, the mineralocorticoid hormone plays a passive role by maintaining the life of adrenalectomized rats.

The acute character of the arthropathies may suggest some infectious etiology; the lesions resemble those observed after injection of a PPLO contaminated tumor exudate (8). However, we have been unable to transfer the disease or to prevent its development with antibiotics, such as terramycin and neomycin (10). Should a microorganism be involved, it would be difficult to explain the fact that the arthritic lesions develop only in animals receiving both STH and LTH treatment.

Acknowledgments

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NOTES AND COMMENTS

EFFECT OF AN ALDOSTERONE ANTAGONIST ON THE FLUID RETENTION OF AMINONUCLEOSIDE NEPHROSIS

ABSTRACT

4-Androsten-3-one-17-spirolactone an antagonist of aldosterone at the kidney tubule level, which possesses a very weak if any glucocorticoid potency, promoted the loss of edema and ascites in intact aminonucleoside nephrotic rats without affecting their increased rate of secretion of aldosterone. The data suggest that in aminonucleoside nephrosis the increased rate of secretion of aldosterone is one of the main factors operating in fluid retention.

A nephrotic syndrome induced in immature rats by daily subcutaneous injections of aminonucleoside has been reported by several groups of investigators (1, 2, 3, 4). In this laboratory special emphasis has been placed on the investigation of two major features of this experimental nephrosis, namely fluid retention and hyperaldosteronism. Within the framework of a larger study undertaken in order to assess changes in the rate of secretion of aldosterone in relation to fluctuation of edema and ascites, normal and aminonucleoside nephrotic rats were studied following short term administration of 4-Androsten-3-one-17-spirolactone (Searle: S. C.-5233).

Recent findings have indicated that this compound blocks the mineralocorticoid activity of exogeneous desoxycorticosterone and aldosterone when administered to adrenalectomized rats, and antagonizes the pressor action of desoxycorticosterone acetate (5).

METHODS

Immature male rats of the Long Evans strain weighing between 75 and 85 gm. were used. They were fed on a stock diet of Purina Fox Chow and received tap water *ad libitum*.

Nephrosis was induced by daily subcutaneous injections of 6-dimethylaminopurine, 3-amino-d-ribose (1.75 mg./100 gm. body weight) administered in 0.2 ml. of water for 8 consecutive days.

SC-5233 was injected for 5 consecutive days at the daily dose of 2.5 mg./100 gm. of body weight in 2 subcutaneous injections of corn oil given 8–10 hours apart. In nephrotic animals, the injections of this compound were begun 72 hours after the last injection of aminonucleoside of puromycin. Adrenal vein blood was collected from the left adrenal gland 16–18 hours after the last injection of 17-spirolactone. Pooled adrenal vein blood from 3–5 rats was extracted according to the method of Bush (6). Dry residues were subjected to one single chromatography in the system benzenemethanol-water (7) following which corticosterone was quantitatively measured by spectrophotometry at 2400 Å and aldosterone by bioassay (8). These methods have been described in detail elsewhere (4). Total serum protein, serum albumin, and globulin were measured by the method of Gornall *et al.* (9), cholesterol by the Liberman-Burchard's reaction.

RESULTS

Previous studies (4) have established that the rate of secretion of aldosterone and corticosterone in the adrenal vein blood of normal control rats is respectively 30 ± 8 (S.D.) μg. of DOCA equivalent and 90 ± 8 μg/kg. of body weight/adrenal/hour. Sixteen hours and 5 days after the last of 8 injections of aminonucleoside of puromycin, untreated nephrotic animals present a marked increase of aldosterone production (at 16 hours, 159 ± 67 and at 5 days, 181 ± 72 μg./kg./body weight/adrenal/hour) and a moder-

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TABLE 1. ALDOSTERONE AND CORTICOSTERONE IN THE ADRENAL VEIN BLOOD OF NORMAL AND NEPHROTIC RATS TREATED WITH SC. 5233

	No. rats in pool	Mean wt. (gm.)	Mean adrenal wt./kg. body wt. (mg.)	Mean Blood vol./adr./kg. (ml.)	μg./kg./adr./hr.	
					Aldosterone (μg. DOCA equiv.)	Corticosterone
Normal rats	3	104	70	18	33	70
	3	110	85	23	55	70
	4	108	83	18	54	100
	4	118	82	20	110	68
Mean ± S.D.					64 ± 33	77 ± 15
Nephrotic rats	3	117	81	20	141	179
	3	122	91	20	219	133
	3	116	87	17	164	173
	3	106	89	19	155	141
	3	106	90	22	72	132
Mean ± S.D.					150 ± 54	151 ± 23

ate but significant rise of corticosterone secretion (at 16 hours, 140 ± 41.5 and at 5 days, 172 ± 41 μg./kg./body weight/adrenal/hour). From 7–12 ml. of ascitic fluid may be aspirated from the abdominal cavity of these animals at the time of laparotomy.

The effect of SC-5233 on the secretion of aldosterone and corticosterone in the adrenal vein blood of four groups of normal rats is presented in Table 1. The mean secretion of corticosterone is equal to 77 ± 15 μg./kg./adrenal/hour. The secretion of corticosterone by SC-5233 treated rats is not significantly different ($P = .1$) from that of normal untreated animals. Of the four groups studied only one presented a moderate increase of aldosterone secretion. The mean value of aldosterone secretion of these four groups is equal to 64 ± 33 μg./kg./adrenal/hour. There is no statistical difference between the aldosterone secretion of SC-5233 treated rats and that of normal untreated controls.

After SC-5233 treatment the rates of secretion of both corticosterone (151 ± 23 μg./kg./adrenal/hour) and aldosterone (150 ± 54 μg./kg./adrenal/hour) of the nephrotic animals were not significantly different ($P < .1$) from those of untreated nephrotic rats. At the time of operation the nephrotic animals presented only traces of ascites. The weight of their adrenals was not appreciably different from that of untreated nephrotic rats (Table 1).

Treatment of nephrotic animals with SC-5233 was followed by an increase of total serum protein and serum albumin to values which were not statistically different from that observed in normal rats but the albumin-globulin ratio (1.1) remained close to that of untreated nephrotic animals (0.9). The level of serum cholesterol (180 ± 123 mg.%) had not returned to values found in normal rats (63 ± 9 mg.%) but was statistically lower ($P < .001$) than that of untreated nephrotic animals, observed at the same period after the end of injections of puromycin (394 ± 107 mg.%) (Table 2).

COMMENT

The present results show that at the dose level of 2.5 mg./100 gm. body wieght/day for 5 days, SC-5233 did not appreciably affect the secretion of aldosterone nor that of corticosterone by either normal or nephrotic animals. Thus, the steroid did not alter

TABLE 2. BIOCHEMICAL DETERMINATIONS ON THE SERUM OF NORMAL AND NEPHROTIC RATS

	Serum protein gm.%	Serum albumin gm.%	A/G ratio	Serum cholesterol mg.%	Ascitic fluid ml./kg. body wt.
Normal rats (9)	5.4 ± .7*	3.4 ± .1	1.7	62.9 ± 9.2	Nil
Untreated nephrotic rats (10)	3.7 ± .7	1.7 ± .6	0.9	394.0 ± 107.0	69.4 ± 21.1
Nephrotic rats + SC-5233 (6)	5.5 ± .7	2.9 ± .5	1.1	180.0 ± 123.5	Traces

Bracketed numbers indicate the number of determinations.
* Standard Deviation of the Mean.

the increased rate of secretion of aldosterone which is one of the major features of aminonucleoside nephrosis (2,4).

The lack of action of SC-5233 on the weight of the adrenals and on the rate of secretion of corticosterone in both the normal and the nephrotic series afford further proof that this compound is devoid of glucocorticoid activity and that the mechanism of its action in improving the state of fluid retention of aminonucleoside nephrosis, is basically different from that of cortisone (4). If in the interpretation of these results one admits that the primary action of SC-5233 is to cancel the effect of hyperaldosteronism at the kidney tubule level, one implies that in aminonucleoside nephrosis the increased rate of secretion of aldosterone is one of the main factors operating in fluid retention. Direct evidence in support of this view was obtained by demonstrating that adrenalectomized aminonucleoside nephrotic rats receiving maintenance doses of aldosterone and corticosterone do not present fluid retention; whereas they do present fluid retention upon administration of aldosterone at doses equivalent to the amount of this hormone secreted by the adrenals of intact nephrotic animals (10).

Although hematocrit determinations have not been performed it is suggested that hemoconcentration might explain the increase of total serum protein and serum albumin of SC-5233 treated nephrotic animals toward normal values.

The observation that this increase was not accompanied by a comparable increase in the albumin-globulin ratio to normal value (1.7) would lend support to this hypothesis.

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BIOLOGICAL POTENCY OF 3,3′,5′ TRIIODOTHYRONINE IN BIRDS

ABSTRACT

3,3′,5′ triiodo-DL-thyronine was less potent than L-thyroxine when tested by the goiter-prevention and inhibition of thyroid I^{131} uptake in birds, and this result is in agreement with similar findings in mammals, and in contrast to the reports that 3,5,3′ triiodo-L-thyronine is more potent in mammals and no more or less potent in birds. 3,3′,5′ triiodo-DL-thyronine was more potent in the inhibition of I^{131} uptake test than in the goiter-prevention test, and in this case no species difference exists between birds and mammals. It was suggested that the higher potency in the I^{131} uptake test might be due to rapid deiodination of 3,3′,5′ triiodothyronine.

It has been well established that 3,5,3′ triiodothyronine is more potent than thyroxine when assayed in a wide variety of biological tests in mammals (1, 2, 3). However, when the two compounds were compared by similar tests in birds, 3,5,3′ triiodothyronine has proven to be no more, and in some cases less, potent than thyroxine (4–7). Another triiodinated thyronine, 3,3′,5′ triiodothyronine, has been shown to be less potent than thyroxine in mammals (8–11), and it seemed of interest to test this compound in birds to ascertain if a species difference in its potency exists between birds and mammals.

MATERIALS AND METHODS

3,3′,5′ triiodo-dl-thyronine[1] (3,3′,5′ T-3), 3,5,3′ triiodo-L-thyronine (3,5,3′ T-3) and L-thyroxine (T-4) were compared in goiter-prevention tests and in tests on their inhibitions of I^{131} uptake by the thyroid gland in single-comb, White Leghorn cockerels. On the 10th day of age, groups of at least 8 birds were started on 0.1% propylthiouracil in the drinking water. The next day, daily subcutaneous injections of at least 3 dosage levels of each compound were begun and given for 10 days while propylthiouracil water was continued. On the 21st day of age, the birds were killed and the thyroid gland and body weights obtained. All three compounds were dissolved in a minimum amount of 0.02 N NaOH and diluted to the desired volume with distilled water and solutions were stored at 2–4° C between injections. Each injection was given in the back of the neck in a volume of 0.2 cc. Commercial chick food was given *ad libitum*. Exactly the same procedure was used on birds from the same hatch to study inhibition of I^{131} uptake except that propylthiouracil was not administered, and in addition, 2 μc of I^{131} were given at the time of the last injection of thyroxine or its analogue. The 24-hour thyroidal accumulation of I^{131} was determined by counting the thyroid glands against suitable standards of the I^{131} used for injection in a well-type scintillation counter. Estimation of potencies of 3,3′,5′ T-3 and 3,5,3′ T-3 in terms of T-4 was done by the parallel-line method (12). The doses used were: T-4, 1.5 and 3 μg.; 3,5,3′ T-3, 0.75 and 1.5 μg.; 3,3′,5′ T-3, 1.5, 3, 6, 20, 40, and 80 μg.

RESULTS

The estimated potency in goiter-prevention tests of 3,3′,5′ T-3 was 0.8, and of

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[1] 3,3′,5′ triiodothyronine was generously contributed by Dr. R. L. Kroc, Warner-Lambert Research Institute.

3,5,3′ T-3, 70, considering T-4 to be 100. Similarly, in inhibition of I^{131} tests, the estimated potency of 3,3′,5′ T-3 was 11, and of 3,5,3′ T-3, 72.

DISCUSSION

In agreement with previous findings, 3,5,3′ T-3 was less potent than thyroxine in birds when tested by the goiter-prevention test (4–7). Since 3,5,3′ T-3 has proven to be more potent than T-4 by the same test in mammals (1–3), a species difference in the activity of these two compounds exists. The reason for this species difference is not known, although it has been suggested that the lack of thyroxine binding protein in the blood of fowl may, in part, explain the discrepancy in results (13). On the other hand, there is evidence in man (14, 15) that alterations in the binding of thyroxine in serum do not alter the potency of this hormone so that the above suggestion can, at best, only be a partial explanation. In general, the trihalogenated thyronines and their analogues have proven to be more potent than similar tetrahalogenated compounds in all classes of vertebrates tested (3, 16), except birds (6) provided the three halogens are in the 3, 5, and 3′ positions. However, when the 3,3′,5′ positions are halogenated, these analogues are less potent than thyroxine (8–11) in mammals, and so it is in the present case when tested in birds.

The present finding in birds that 3,3′,5′ T-3 is approximately 14 times more potent when tested by inhibition of I^{131} than when tested by the goiter-prevention method is in agreement with similar findings in mammals (8–10). It is not clear why 3,3′,5′ T-3 is more potent in inhibition of I^{131} uptake than in inhibition of thyroid enlargement in goitrogen-treated animals when both tests presumably measure inhibition of thyroid stimulating hormone release by the pituitary. If the finding (17) that this compound is rapidly deiodinated *in vivo* in man were to apply as well to birds this might suggest that iodide is liberated and that the iodide then interferes with the thyroidal accumulation of I^{131}. This is a suggestion that can be tested by experimentation, such as a measure of the rate of deiodination of 3,3′,5′ T-3 in birds and then testing equivalent amounts of injected iodide on I^{131} uptake. Although the significance of the discrepancy between goiter-prevention and inhibition of I^{131} estimates of potency remains unknown, it is clear that this discrepancy exists in birds as well as in mammals.

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PRODUCTION OF ADRENAL ATROPHY BY 6-METHYL-17-ACETOXYPROGESTERONE, WITH REMARKS ON THE ADRENAL EFFECTS OF OTHER PROGESTATIONAL AGENTS

ABSTRACT

A daily oral dose of 2 mg. of 6-methyl-17-acetoxyprogesterone to male rats and 1.5 mg. to females produced a depression of adrenal weight after two weeks. Atrophy was more marked in intact females (55% depression of weight) than in males (21%). In spayed females a subcutaneous dose of 1 mg. per rat per day for two weeks produced a 70% decrease; in the same test 1 mg. doses of progesterone and 17-(2-methallyl)-19-nortestosterone produced 20% and 14% depressions respectively, whereas neither 17α-ethynyl-17-hydroxy-5(10)-estren-3-one, nor 17-ethynyl-19-nortestosterone, nor 17-acetoxyprogesterone had any significant adrenal weight effect. A cold-shock test in mice after 2 weeks of daily injections of 500 μg. of the 6 methyl derivative suggested that adrenal function was impaired.

6-Methyl-17-acetoxyprogesterone is a potent, orally active, progestational agent in animal testing; subcutaneously it is about 50 times more potent than progesterone in a Clauberg test (Elton, unpublished). Toxicity studies in this laboratory indicated that this material caused a marked adrenal atrophy when administered to rats for two weeks. In view of this, it was surprising that little corticoid activity could be demonstrated: Saunders (unpublished) showed that the compound had only 5–10% of the activity of cortisone acetate in a liver glycogen deposition test, whereas Hershberger (unpublished) found no anti-inflammatory activity in preliminary rabbit iritis and rat foot edema tests. To the best of our knowledge this is the first progestin to produce major decrease in adrenal weight. In a study on the adrenal weight effects of various pregnane derivatives Huggins and Jensen (1) found the strongest inhibitor to be 9α-fluoro-11β-hydroxyprogesterone, which is a potent corticoid. In view of the fact that 6-methyl-17-acetoxyprogesterone is now receiving clinical evaluation, we studied this material further, together with various progestoids that are now receiving clinical attention.

In our toxicity study the hormone was administered orally in food, available *ad libitum* for two weeks (Table 1). The treated females showed a markedly decreased adrenal size when compared with female controls. These proved to be the smallest adrenal glands of any group of rats of comparable size ever studied in this laboratory. The treated males had smaller adrenals than corresponding controls, although here body growth was also depressed.

We confirmed these observations in an additional experiment using 40-day-old female rats that had been spayed 10 days earlier. 6-Methyl-17-acetoxyprogesterone was compared with a series of the newer progestoids (Table 2). All compounds were administered subcutaneously for 14 days at a daily dose of 1 mg. Progesterone and 17α-(2-methallyl)-19-nortestosterone caused slight, but statistically significant, inhibition of adrenal growth when compared with oil-injected controls Again 6-methyl-17-acetoxyproges-

TABLE 1. EFFECTS OF 6-METHYL-17-ACETOXYPROGESTERONE ADMINISTERED ORALLY, MIXED WITH FOOD, ON BODY WEIGHT, GROWTH, FOOD INTAKE AND ADRENAL WEIGHTS OF INTACT ADULT RATS; EACH GROUP CONTAINED 6 ANIMALS

Sex	Treatment	Dosage, mg./rat/day	Final body weight gm.	Weight gain gm./rat/day	Food intake, gm./rat/day	Adrenal weight, mg.
Male	Control	0	316	5.31	24.63	44.7
	Treated	2.05	288*	4.93	20.46	36.5*
Female	Control	0	194	2.40	15.93	50.3
	Treated	1.48	196	2.05	14.85	22.6*

* Mean of treated group differed significantly from that of the corresponding control with $P < 0.05$.

TABLE 2. EFFECTS OF VARIOUS PROGESTATIONAL AGENTS ON ADRENAL WEIGHT IN SPAYED RATS. EACH MATERIAL WAS ADMINISTERED AT A DOSE OF 1 MG.

Test material	N	Body wt.	Adrenal wt.
Oil	9	193	58.0
17α-Ethynyl-17-hydroxy-5(10)-estren-3-one	9	149	57.8
17-Ethynyl-19-nortestosterone	9	161	54.3
17-(2-Methallyl)-19-nortestosterone	9	190	49.7*
Progesterone	9	190	46.7*
6-Methyl-17-acetoxyprogesterone	9	185	17.2*

* Mean differs significantly from oil control with $P < 0.05$.

terone produced an extreme adrenal atrophy, whereas neither 17α-ethynyl-17-hydroxy-5(10)-estren-3-one, nor 17α-ethynyl-19-nortestosterone, caused any significant decrease in adrenal size.

In a third study 6-methyl-17-acetoxyprogesterone was compared, at several dose levels, with 17-acetoxyprogesterone and with progesterone. 6-Methyl-17-acetoxyprogesterone appeared to produce a depression of adrenal weight that was linear over a range of doses from 30 to 1000 μg. per rat per day (Figure 1). Neither progesterone nor 17-acetoxyprogesterone caused significant change from control.

In an attempt to determine whether these smaller adrenals were also depressed functionally, treated male mice were challenged with cold shock. Five hundred μg. of 6-methyl-17-acetoxyprogesterone or 17-acetoxyprogesterone were injected subcutaneously each day for two weeks. Twenty-four hours after the final injection the animals were placed in a cold room that varied from 4 to 6° C. Deaths were recorded at hourly intervals throughout the day. The treated groups were compared with intact controls and controls that had been adrenalectomized two days before the test. All controls had received daily injections of solvent. None of the intact group died during the $8\frac{1}{2}$ hours the mice were in the cold room, whereas all adrenalectomized mice were dead within 4 hours. About half of the 6-methyl-17-acetoxyprogesterone-treated animals succumbed (Table 3). 17-Acetoxyprogesterone had no significant effect. These data suggest that adrenal function, like size, is depressed by 6-methyl-17-acetoxyprogesterone.

COMMENT

The potent progestational agent, 6-methyl-17-acetoxyprogesterone, produced marked adrenal atrophy and, apparently, some depression of adrenal function in laboratory animals after two weeks of treatment. Progesterone and 17-(2-methallyl)-19-nortestosterone produced a slight depression of adrenal weight in one experiment; however, progesterone was ineffective in a second study. Whether prolonged treatment with hu-

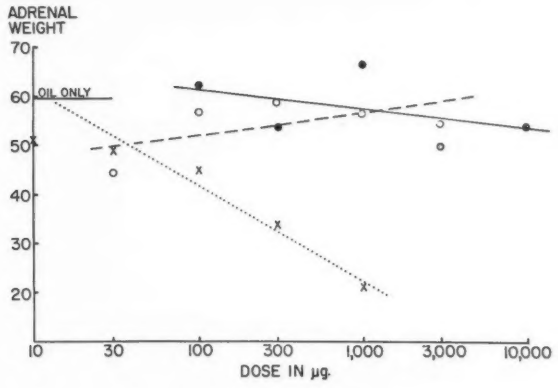

FIG. 1. Effects of progesterone, 17-acetoxyprogesterone, and 6-methyl-17-acetoxyprogesterone on adrenal weight in spayed female rats. Each point represents the mean of 6 rats. Compounds were administered subcutaneously each day for two weeks. Only 6-methyl-17-acetoxyprogesterone produced a curve with a slope significantly different from zero. Regression formulae and key:

Progesterone ●—● adrenal weight = 69.4 − 4.0 log dose
17-Acetoxy-progesterone ○- -○ adrenal weight = 43.4 + 4.4 log dose
6-Methyl-17-acetoxyprogesterone ×···× adrenal weight = 79.5 − 18.9 log dose.

TABLE 3. EFFECTS OF 6-METHYL-17-ACETOXYPROGESTERONE AND 17-ACETOXYPROGESTERONE ON SURVIVAL OF MALE MICE EXPOSED TO LOW TEMPERATURES (4–6° C)

Treatment	Deaths	Median death time
Controls, Intact	0/10	—
Controls, Adrenalectomized	15/15	3 hrs.
17-Acetoxyprogesterone	0/10	—
6-Methyl-17-acetoxyprogesterone	4/7*	8½ hrs.

* Death rate differs significantly from that of intact controls, with $P < 0.05$ (Fisher's exact test for 2×2 tables).

man progestational doses of 6-methyl-17-acetoxyprogesterone will produce toxicity remains to be evaluated.

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THE DURATION OF ACTIVITY OF THE BENZILOYL HYDRAZONES OF TESTOSTERONE-17-HEPTANOATE, ESTRONE-3-HEPTANOATE AND 17α-HYDROXY-PROGESTERONE-17-HEPTANOATE[1]

ABSTRACT

The benziloyl hydrazones of testosterone-17-heptanoate, estrone-3-heptanoate and 17α-hydroxyprogesterone-17-heptanoate gave prolonged hormonal effects after subcutaneous injection into rats when compared to equivalent doses of the parent hormones, their benziloyl hydrazones or their heptanoate esters.

Carboxylic acid esters of many steroids have been widely used for replacement therapy because of their tendency to prolong hormonal activity when administered by injection. In addition to the ester grouping, a necessary requisite for activity in all of these derivatives is an oxygen function, almost always in the form of a ketone (or its enol variant), located elsewhere on the steroid molecule. Any attempt to replace this oxygen by a nitrogen derivative has invariably resulted in a marked diminution of activity. For example, the 3-thiosemicarbazone of testosterone is reported as having 1/40 the activity of the corresponding 3-keto compound (1) and testosterone-3-oxime-17-propionate is less than 1/20 as potent as testosterone-17-propionate (2). This report concerns the changes in the intensity and duration of hormonal effects which result when the keto function of certain steroids is replaced by the benziloyl hydrazone grouping.[2]

MATERIALS AND METHODS

Androgens

Forty-day-old male rats weighing 125 ± 15 gm. were castrated. Thirty days later they were injected subcutaneously with the compound dissolved in 0.5 ml. of corn oil. Doses were all equivalent to 14.4 mg. of testosterone. Animals were sacrificed at weekly intervals and prostate, seminal vesicles, levator ani and carcass weighed. The seminal vesicles were emptied before weighing and the prostate included the coagulating gland. Four rats were used for each sacrifice time period. The following substances were assayed in this manner: testosterone, testosterone-17-heptanoate, testosterone-3-benziloyl hydrazone and testosterone-17-heptanoate-3-benziloyl hydrazone.

Estrogens

Ovariectomized rats proven to be reactors by previous priming with standard estrone 5 μg. in 0.2 ml. corn oil were used. After resting one week they were dosed subcutaneously with the compound dissolved in 0.2 ml. corn oil. Doses were equivalent to 550 μg. estrone per rat. Vaginal smears were made daily and examined for evidence of estrus. Twenty animals were used for each compound. The following substances were assayed in this manner: estrone, estrone-3-heptanoate, estrone-17-benziloyl hydrazone and estrone-3-heptanoate-17-benziloyl hydrazone.

Gestagens

Progestational activity was measured not by the usual McPhail-Clauberg test using young rabbits but by a technique described by Junkmann (3) which is based on the antiluteinizing action of progesterone. Immature female rats weighing 50 ± 5 gm. were given estradiol valerate 10 μg. intramuscularly. At the same time the compound under study was given subcutaneously. There were 10 animals to a group. After 8 days the animals were killed and the number of corpora lutea counted. A similar series was allowed to run for 14 days. Control animals received only the estrogen. To indicate the activity of progesterone itself one

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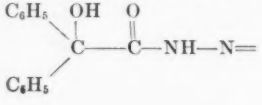
[1] Presented in part at the First Annual Meeting of the Canadian Federation of Biological Societies, Kingston, Ontario, June, 1958.

[2] The structure of the benziloyl hydrazone grouping is



group of animals was given daily 1 mg. doses of progesterone. The experimental compounds tested were 17α-hydroxyprogesterone-17-heptanoate and 17α-hydroxyprogesterone-17-heptanoate-3-benziloyl hydrazone given in doses equivalent to 10 mg. of progesterone.

RESULTS

Androgens

The effect on the growth of the prostate and seminal vesicles is shown in Figures 1 and 2 respectively. Testosterone-17-heptanoate reached a peak 4 weeks after administration and persisted until the 8th week. By comparison, the 3-benziloyl hydrazone of testosterone-17-heptanoate showed a slower onset of activity and did not reach a maximum effect until 6 weeks but its peak was slightly higher and it was effective for about 14 weeks. It is noteworthy that the 3-benziloyl hydrazone of testosterone without the ester substituent was completely ineffective. As expected, free testosterone gave an androgenic response but of very short duration.

Estrogens

In this series all of the substances showed strong and almost equal estrogenic activity for the initial week of the test (Fig. 3). However, those animals which had received estrone and estrone-17-benziloyl hydrazone came out of estrus quite rapidly. As with testosterone-17-heptanoate, the use of the benziloyl hydrazone of estrone-3-heptanoate showed a marked prolonging effect. Thus estrus persisted for 8 weeks in 50% of the animals treated with estrone-3-heptanoate as compared with 12 weeks for estrone-3-heptanoate-17-benziloyl hydrazone and even beyond this period the rate of fall-off was much slower.

Gestagens

It appears that a single injection of 17α-hydroxy-progesterone-17-heptanoate-3-

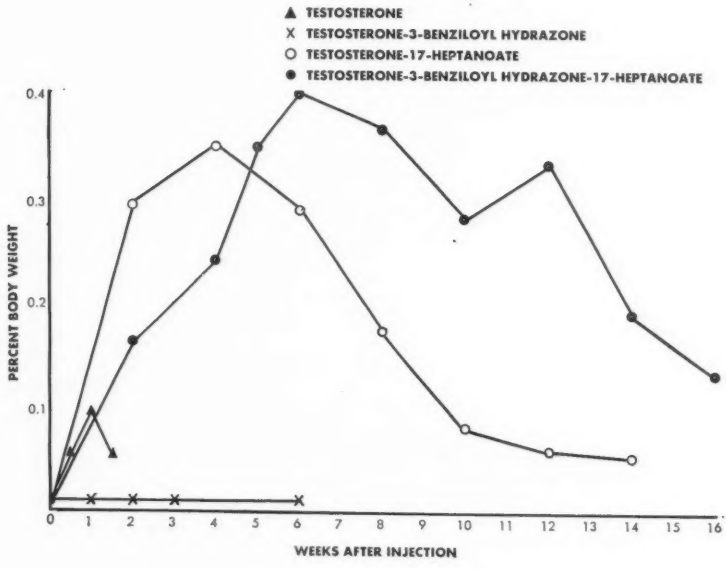


FIG. 1. Growth response of prostate expressed as per cent of body weight.

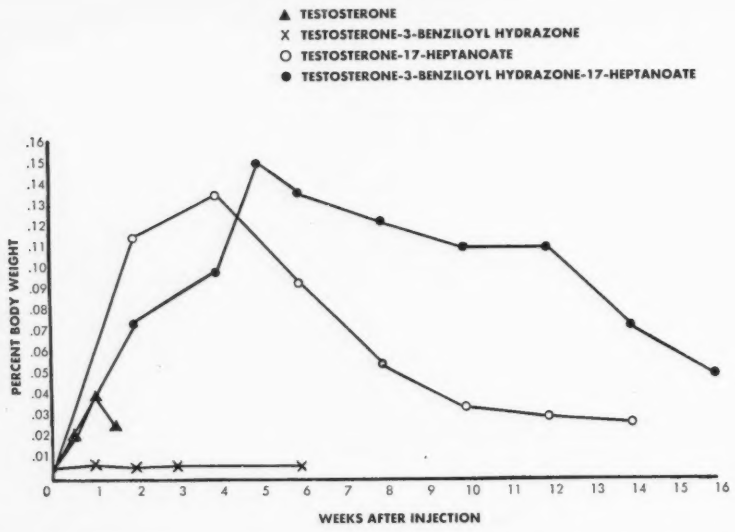


FIG. 2. Growth response of seminal vesicles expressed as per cent body weight.

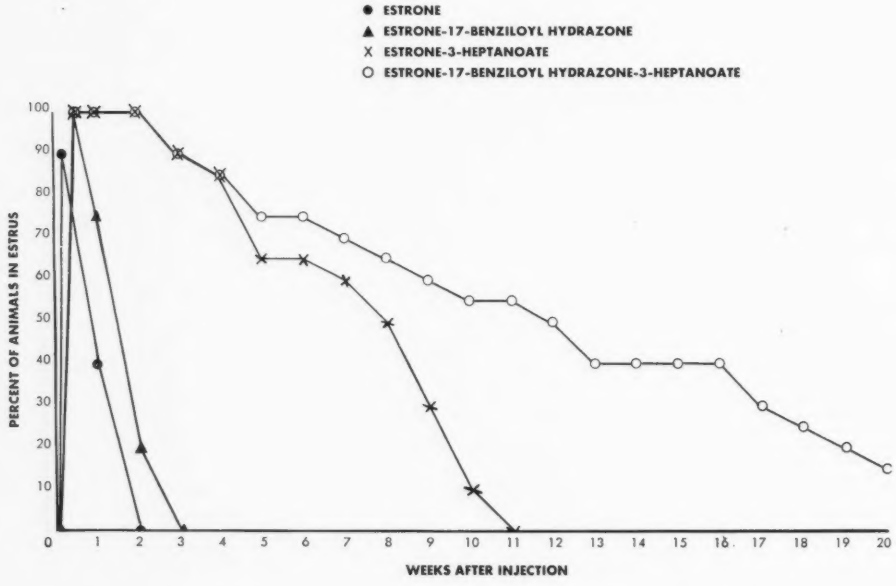


FIG. 3. Estrogenic response expressed as per cent of animals in estrus.

TABLE 1. ANTILUTEINIZING EFFECT OF PROGESTERONE PREPARATIONS

Treatment	Average number of corpora lutea per animal*	
	8 days	14 days
Control	5.4	—
Progesterone†	0.2	—
17-α-hydroxyprogesterone-17-heptanoate‡	2.3	3.0
17-α-hydroxyprogesterone-17-heptanoate-3-benziloyl hydrazone‡	1.3	4.1

* Ten animals were used in each group.
† One mg. daily injected subcutaneously.
‡ Single subcutaneous injection equivalent to 10 mg. progesterone.

benziloyl hydrazone is slightly more effective in suppressing corpora lutea formation for a period of 8 days than an equivalent amount of the ester itself and it is not quite as effective as the equivalent amount of progesterone given in daily doses (Table 1). After 14 days the activity has fallen off and there is little difference between the ester and its hydrazone.

DISCUSSION

Without tissue and excretion studies it would be idle to speculate as to the influence of the benziloyl hydrazones on the activity of the steroid esters. Protracted effect may be due to slow absorption and release from the injection site because of the poor solubility of these derivatives. Junkmann (4) feels that retarded absorption of steroid esters plays only a minor role in imparting prolonged hormonal activity. He contends that the major mechanism involves a retardation in the rate of metabolic transformation, and hence, in the rate of elimination of the esterified steroid from the body. In the case of the benziloyl hydrazones of the steroid esters, this mechanism may be further complicated by the need for preliminary hydrolysis of the hydrazone grouping to a free keto group. In any case the high order of activity of the heptanoates of testosterone-17- and estrone-3-benziloyl hydrazone as compared with the very low activity of the corresponding non-esterified compounds strongly suggests a complementary relationship between the ester and hydrazone substituents.

Acknowledgments

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EFFECT OF ALDOSTERONE-ANTAGONIST, SC 8109, ON THE SECRETION OF ALDOSTERONE IN NORMAL RATS

ABSTRACT

The effect of aldosterone-antagonist, SC 8109, on the aldosterone content of the adrenal vein blood of rats was determined in animals fed normal and low salt diets.

When the animals were fed normal diets, SC 8109 had no effect on the aldosterone secretion rate either after a single injection or after a week of continuous treatment.

SC 8109 given continuously for a week to animals which were fed a low salt diet at the same time produced a dramatic increase in the aldosterone secretion rate above that obtained with diet alone.

It is concluded that if this material acts by blocking the effect of aldosterone on the kidney, aldosterone must play an important physiological role in sodium deficiency but not in the presence of adequate salt intake and intact adrenal glands.

Several new synthetic steroid lactone compounds have been reported to inhibit the action of aldosterone on the kidney. The action has been demonstrated as an inhibition of the effect of this hormone or desoxycorticosterone on the urinary sodium-potassium ratio of adrenalectomized rats (1), and on urinary sodium excretion in humans (2). We have studied the effect of one of these compounds, SC 8109 (Searle), on the rate of aldosterone secretion into the adrenal vein blood of rats.

METHODS

Adrenal vein blood was collected as previously described (3). Male albino rats weighing approximately 200 g. were used. Minor modifications include a reduction of the bleeding period from 1.5 to 1.0 hours and the elimination of the use of atropine.

A neutral ethyl acetate extract of the blood was chromatographed in two solvent systems for the isolation of aldosterone as described by Neher and Wettstein (4). Aldosterone was estimated quantitatively by the soda fluorescence reaction on the second chromatogram by a specially constructed fluorimeter (5). The blood of several rats was pooled for each experiment. The quantity of aldosterone released into the adrenal vein is expressed as μg./kg./adrenal/hour. In the earlier experiments the test material was dissolved in corn oil and injected subcutaneously. In later experiments, the solvent mixture propylene glycol, benzyl alcohol, distilled water (5:2:3) was used. There was no evidence in any of the experiments that these solvents affected the aldosterone secretion rate. The low salt diet used in these studies was prepared according to Cotlove *et al.* (6).

RESULTS

The effect of a single subcutaneous injection of 400 μg. of SC 8109 was studied in two experiments. The material was injected two hours before adrenal vein blood collection was started. The aldosterone secretion rate was 0.298 μg./kg./adr./hr. in the first experiment and 0.494 in the second. This treatment was apparently without effect as the values are within the range obtained in untreated animals. In eight different experiments in untreated rats the mean aldosterone secretion rate was 0.410 μg./kg./adr./hr. (S.D. ±0.201). Urinary sodium-potassium ratios of the SC 8109-treated animals in the two-hour period between injection and collection of blood were slightly, but not significantly, raised above those of untreated animals studied at the same time.

The effect of 5 mg. of SC 8109, injected 3 or 5 hours before collection of adrenal vein blood was started, was determined on three occasions. The mean aldosterone secretion rate was 0.376 μg./kg./adr./hr. (individual estimates 0.411, 0.488 and 0.228).

The effect of continuous treatment with 10 mg./day of SC 8109 (in two divided doses) for one week was studied. Four experiments were performed. The last injection was given 6 hours before collection in the first experiment and 2 hours before in the others. The mean aldosterone secretion rate in these experiments was 0.519 μg./kg./adr./hr. (individual estimates 0.454, 1.230, 0.144 and 0.250).

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The effect of single and continuous injections of SC 8109 on animals given low sodium chloride diets was then studied. In five experiments with diet alone, for one week, the aldosterone secretion rate was 0.578 μg./kg./adr./hr. (individual estimates 0.289, 0.594, 0.905, 0.362 and 0.740) as compared with 0.410 obtained with rats on normal diet.

Four experiments were performed in which the animals received the low salt diet for one week and a single injection of 5 mg. of SC 8109 followed by collection of adrenal vein blood 3 or 5 hours later. The mean aldosterone secretion rate was 0.745 μg./kg./adr. hr. (individual estimates 1.26, 0.81, 0.324 and 0.586).

In two experiments, the animals received 10 mg./day of SC 8109 (in two divided doses) for one week during which time they were fed a low salt diet. The mean aldosterone secretion rate was 1.845 μg./kg./adr./hr. (individual estimates 1.7 and 1.99). This value is about the same as that obtained in two other experiments in which the animals were given a low salt diet for two weeks but no SC 8109. In this case the mean aldosterone secretion rate was 1.795 μg./kg./adr./hr. (individual estimates 2.1 and 1.49).

The results obtained in all of these experiments have been illustrated in Figure 1.

COMMENT

Most of the reports on the effect of SC 8109 support the hypothesis that this substance inhibits the effect of aldosterone on the kidney. One report, however, does sug-

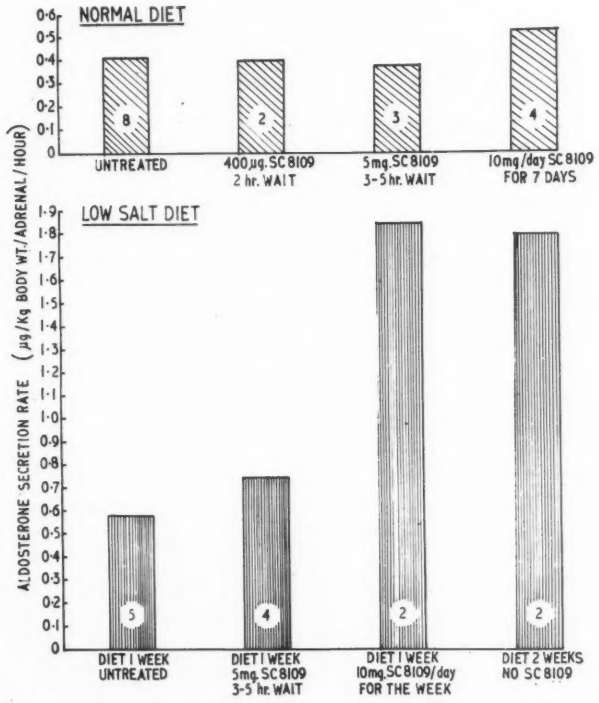

FIG. 1. Aldosterone in adrenal vein blood of rats. Each column represents the mean value obtained in several similar experiments. Each experiment represents a single determination obtained by pooling the blood of (usually) three rats.

gest that the effect of this drug cannot be explained entirely in this manner (7).

If the theory that the steroid blocks the action of aldosterone on the kidney is correct, then it can be assumed that it must have the same effect on endogenously produced aldosterone. One would expect that, if aldosterone plays an important physiological role, blocking the action of the hormone would result in a compensatory increase in the production of aldosterone by the adrenal gland. In the present study, neither single injections nor continuous treatment with the compound for one week resulted in a consistent increase in the secretion rate of aldosterone when the animals were fed normal diets. On the other hand, when the animals were fed a low salt diet for one week and treated with SC 8109 at the same time, aldosterone levels increased dramatically, reaching those found in animals fed the low salt diet alone for two weeks. The fact that there was a moderate increase in the aldosterone secretion rate in the sodium deficient animals after a single injection of SC 8109 suggests that it was probably not necessary to give continuous treatment for the entire week of sodium depletion in order to demonstrate a significant effect.

These results lead to the conclusion that in the rat under normal conditions, i.e. when there are adequate amounts of corticosterone and dietary salt, aldosterone must play a relatively unimportant role. However, the positive response noted in sodium deficiency would emphasize once again the importance of sodium abundance and deficiency on the rate of secretion of aldosterone by the adrenal gland.

The present experiments support the work of Salassa *et al.* (8) who showed, on a patient with primary aldosteronism, that the compound had a marked effect on urinary electrolytes only when aldosterone excretion was high. They also support the results of Kagawa *et al.* (1) on adrenalectomized rats and of Liddle (2) on human subjects and dogs and the more recent work of Ross and Bethune (9) in man.

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THE ADSORPTION OF I^{131}-INSULIN TO GLASS[1]

ABSTRACT

Reversible adsorption of I^{131}-insulin to glassware can occur unless adequate amounts of another accompanying protein are present in the solutions. The significance of these phenomena as related to the preparation of insulin standards for micro-insulin assays is discussed.

A requisite for any bioassay is a stable standard. In dilute solutions, such as have been used for standards for micro-insulin assays, large proportions of the I^{131}-insulin are adsorbed to the walls of the container. This has been previously described by Ferrebee, Johnson, Mithoefer and Gardella (1) and by Newerly and Berson (2). Under certain conditions the adsorption to the vessel may become a rapidly reversible reaction. This could be a serious source of contamination of standards or unknown samples to be assayed.

METHODS AND MATERIALS

All the I^{131}-insulin used in these experiments was obtained from Abbott Laboratories, Oak Ridge, Tennessee, and contained 100 μg./ml. Stock solutions for various experiments were made by diluting with acidified saline. Acidified saline was made by diluting 1 ml. concentrated hydrochloric acid to 2 liters with 0.9% sodium chloride. This solution has a pH of approximately 2.5. Radioautographs were made by wrapping Ansco non-screen X-ray safety film around 100 ml. graduated cylinders containing various solutions and allowing them to stand for one or two weeks. The radioactive content of solutions was measured by plating samples in tared stainless steel cups 2.5 cm. in diameter, drying under an infra-red heat lamp, weighing, counting with an end window Geiger tube, and correcting for self absorption. Dilutions of radioactive insulin could be made without loss if the insulin were accompanied by a suitable quantity of another protein (*e.g.* 5% gelatin).

RESULTS

In preliminary experiments it was found that radioactivity was lost from aqueous I^{131}-insulin solutions in pyrex glass containers, in siliconed glassware, and in polyethylene reagent bottles. This is in agreement with the work of Reeve and Franks (3) with albumin-I^{131} and Magnus, Guld, Waaler and Magnusson (4) with various tuberculin preparations.

When a dilution of I^{131}-insulin is made without accompanying protein there is an initial rapid loss of radioactivity from solutions, which takes place before the solution can be mixed and an aliquot portion sampled. This is followed by a slower loss. These findings are in keeping with Bull's report concerning the adsorption of bovine serum albumin on glass (5).

The adsorption of the radioactivity to glass from acidified saline may be seen in the radioautographs (Fig. 1). In the cylinder containing 5% gelatin there was negligible radioactivity above the surface of the solution. After the cylinder was emptied and rinsed, there was negligible radioactivity retained by the glass. This demonstrates the ability of 5% gelatin to confine the radioactivity within the limits of the solution and the relative ease with which the radioactivity in 5% gelatin may be rinsed from the vessel. In the cylinder containing acidified saline radioactivity was found above the surface of the liquid. After the cylinder was emptied and rinsed, radioactivity was found above the former level of the liquid and negligible amounts were found below.

In order to approximate the loss which might be expected in the actual preparation and injection of insulin standards, dilutions of stock I^{131}-insulin (approximately 2 U./

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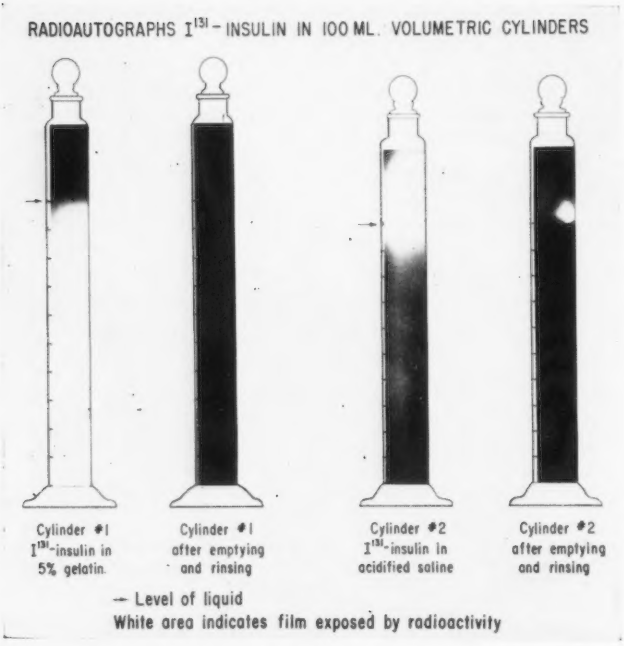
RADIOAUTOGRAPHS I^{131} - INSULIN IN 100 ML. VOLUMETRIC CYLINDERS
Cylinder #1 I^{131}-insulin in 5% gelatin.
Cylinder #1 after emptying and rinsing
Cylinder #2 I^{131}-insulin in acidified saline
Cylinder #2 after emptying and rinsing
— Level of liquid
White area indicates film exposed by radioactivity

FIG. 1. Dilutions of 1:1000 of the stock I^{131}-insulin (100 μg./ml.) were made in two 100 ml. volumetric cylinders. Number 1 contained 5% U.S.P. gelatin in acidified saline and Number 2 contained acidified saline. Both were inverted ten times and stored at about −20° C for four days. X-ray film was wrapped around the cylinders and they were allowed to stand at room temperature for one week. The film was removed. The cylinders were emptied, filled with distilled water, and emptied. The filling with distilled water and emptying were repeated. Unexposed x-ray film was wrapped around the empty cylinders and they were allowed to stand for two weeks. Positive contact prints were pasted on reproductions of the two 100 ml. graduate cylinders used.

ml.) to concentrations of insulin actually used in micro-assays were made. Three 100 ml. graduated cylinders were filled to the 100 ml. mark with acidified saline, 0.01% U.S.P. gelatin in acidified saline, and 5% U.S.P. gelatin in acidified saline. One-tenth ml. of the stock I^{131}-insulin (100 μg./ml.) was removed from the stock vial with each of three one-quarter ml. tuberculin syringes and deposited below the surface of the liquid in the three cylinders. This volume can be delivered with an accuracy of 5% or better. Each cylinder was inverted ten times and allowed to stand at room temperature for one hour. Aliquots were removed from each cylinder and deposited in a suitable volume of 50% gelatin. Aliquots of the 5% gelatin were plated for counting. If the assumption is made that there was no loss in the 5% solution of gelatin, there was a loss of 33% in the acidified saline. Even the low concentration of 0.01% gelatin reduced this loss to 13%.

The following experiment was for the purpose of studying the possibility of contamination by the adsorption to glass and desorption from glass into protein solutions. A sample approximately equal to 0.2 units was removed from a stock I^{131}-insulin supply

with a one-quarter ml. tuberculin syringe. The stock supply was made up in acidified saline (100 μg./ml.). The contents of the syringe was expelled. The syringe was rinsed with tap water three times, rinsed with detergent (Dural H approximately 0.2%) three times, and allowed to soak in detergent for six hours. It was then rinsed three times in tap water, three times in distilled water, and dried. Heparinized dog plasma was drawn up into the syringe for 30 seconds and expelled into a stainless steel cup for counting. The plasma from the syringe contained an amount of radioactivity equivalent to 0.004 μg. of insulin. This is a roughly equivalent to 100 μU.

DISCUSSION

If non-iodinated insulin adsorbs to glassware as does I^{131}-insulin, it is evident that the adsorption can influence the measurements of the insulin content of body fluids by bioassay in at least two ways. First, the instability of the standards would make measurements inaccurate; and secondly, insulin adsorbed to glassware could easily contaminate unknown plasma samples placed in that same glassware.

The above data confirm the previous finding (1, 2) that I^{131}-insulin adsorbs to vessels. In addition, we found I^{131}-insulin can be eluted from vessels by a protein solution. That these phenomena apply as well to nonradioactive insulin will be the subject of another publication (6).

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THE INFLUENCE OF PARATHYROID FUNCTION UPON THE TRANSPORT OF CALCIUM IN ISOLATED SACS OF RAT SMALL INTESTINE[1]

ABSTRACT

In a medium containing physiological amounts of ionized calcium (1.25 mM) inverted sacs of rat small intestine are able to actively transport Ca^{45} from mucosa to serosa against a concentration gradient. Parathyroidectomy 3 or more hours before isolation of the intestinal sac results in a decreased ability of such sacs to develop and maintain a concentration difference between the serosal and mucosal fluids.

It is generally considered that the parathyroid hormone has little or no influence upon the absorption of calcium from the gastrointestinal tract (1). However, Aub *et al.* described a case of hyperparathyroidism in whom the fecal calcium excretion (normal before operation at a time when the serum calcium concentration was increased) rose

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following removal of an adenoma at a time when the serum calcium concentration had fallen (2). Also Albright *et al.* in studying a case of hyperparathyroidism noted a low excretion of fecal calcium at a time when the calcium concentration of the serum was elevated (3). Recently Talmage and Elliott have reported that in the rat removal of the parathyroid gland results, within 2 hours, in a decreased rate of disappearance of Ca^{45} from loops of small intestine *in vivo* (4). The present report describes the effect of parathyroidectomy upon the active transport of Ca^{45} *in vitro* by isolated loops of rat intestine, and the effect of the addition of purified parathormone to this system.

METHODS

Sprague-Dawley rats, weighing approximately 120 gm., were maintained on a low-calcium diet for a period of four to six days. Half of the group were parathyroidectomized four hours before being sacrified by decapitation. The first 3 cm. of the small intestine (starting at the pylorus) were removed, washed in cold isotonic saline, inverted and a small sac prepared by the method of Wilson and Wiseman (5). The solution inside the sac (serosal surface) and that on the outside (mucosal surface) were of the same composition: NaCl, 135 mM, KCl, 11 mM and 10 mM of a pH 7.4 sodium phosphate buffer. Most of the experiments were carried out with a calcium concentration of 1.25 mM and 5 microcuries of Ca^{45} (neutralized) per 100 ml. of solution. A calcium concentration of 0.04 mM was employed in some experiments. For studying the effect of adding hormone *in vitro*, parathormone B prepared by countercurrent distribution (6) was added to a final concentration of 50 units per ml. The hormone was added to the solution both inside and outside the sac. The volume inside the sac was usually 0.6 to 0.75 ml. of solution and did not change significantly during the course of the experiments. After the sacs had been prepared, they were placed in 2.5 ml. of buffer ina a 25 ml. Erlenmeyer flask, gassed with O_2 for 1 minute, and then incubated at 37° in a water bath shaker for ½, 1, 1.5, 2, or 3 hours.

At the end of an experiment the sac was cut open and the solution inside allowed to run into a test tube. The Ca^{45} was counted on a Nuclear-Chicago model D47 gas flow counter with a micromil window and a Nuclear-Chicago scaler with automatic sample changes. The results are plotted as a ratio of the Ca^{45} concentration on the serosal side to that on the mucosal side (S/M).

RESULTS AND CONCLUSIONS

When parathyroidectomy is performed 4 or more hours before the removal of the gastrointestinal tract, the results recorded in Figure 1 are obtained.

At physiological concentrations of calcium in the medium (1.25 mM), the intestinal sac from the normal animal was able to produce and maintain a 3-fold difference in

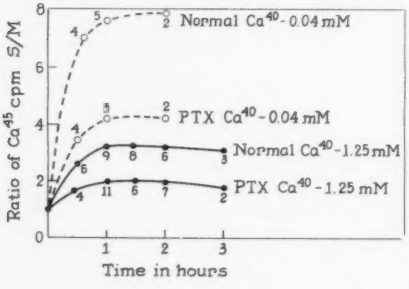

FIG. 1. The effect of parathyroidectomy upon the absorption of Ca^{45} in inverted, isolated sacs of rat small intestine. The ratio (S/M) of the concentration of Ca^{45} inside the sac (serosal surface) is plotted against time for two levels of stable calcium in the bathing medium. The points represent the mean values of the ratio determined in 3 to 11 animals. The numbers indicate the number of animals used to determine each point.

concentration of Ca^{45}, whereas sacs from parathyroidectomized animals could only produce a 2-fold concentration difference. This difference was produced in the first hour and maintained essentially unchanged for the next two hours. The S/M concentration ratios observed after 1 hour in 11 parathyroidectomized animals were 1.5 to 2.5 and in 9 normal animals 2.5 to 4.4. When analyzed statistically the differences were highly significant ($P<0.001$).

When lower concentrations of stable calcium (0.04 mM) were used in the medium, the concentration ratios obtained at the end of 1.5 hours incubation were greater than when

the calcium concentration was 1.25 mM. In 6 normal animals the S/M ratios were 7.6 ± 1.8 and in 5 parathyroidectomized animals 4.2 ± 1.6.

Since it appeared that in medium with physiological concentrations of calcium the ability to concentrate calcium was limited and that equilibrium was reached within 1 to 1.5 hours, one experiment employing 3 normal and 3 parathyroidectomized animals was carried out in which the initial S/M ratio of stable and labelled calcium was 3.0. After 1 hour the mean S/M ratio of Ca^{45} was 3.2 in the sacs from the normal animals and 1.9 in the sacs from the parathyroidectomized animals.

In several experiments the concentrations of stable calcium in the mucosal and serosal fluids were determined. The concentration ratios obtained were similar to those observed with the Ca^{45}; for example, in six normal animals the calcium concentration in the serosal fluid was 1.93 ± 0.22 mM and 0.62 ± 0.16 mM in the mucosal fluid after one hour incubation in a solution with an initial concentration of 1.25 mM. The S/M ratios of Ca^{40} in this group were 2.8 ± 0.4 compared to S/M ratios of Ca^{45} of 2.7 ± 0.5.

The effect of the addition of parathyroid hormone *in vitro* to sacs obtained from parathyroidectomized animals has been studied. In all, 12 animals have been studied. There has been a significant effect after 1.5 hours in 7 of the preparations (mean S/M ratio of 2.7) and no effect in the other 5 (mean S/M ratio 2.1).

DISCUSSION

The present data indicate that there is a mechanism in the small intestine of the rat for actively transporting calcium against a concentration gradient from the mucosal to the serosal surface. From data not presented and from the work of Schachter and Rosen (7), the calcium transporting mechanism appears to be limited to the duodenum and early jejunum. Schachter and Rosen have shown that it is influenced by vitamin D. The present work indicates that parathyroid hormone also influences this transport system. In the absence of vitamin D or parathyroid hormone, the isolated intestine appears to be unable to produce and maintain a normal concentration gradient (S/M) of calcium.

The results obtained to date, when parathyroid hormone is added *in vitro*, have not been consistent enough to warrant any conclusions. Part of the difficulty may be explained by the fact that a molecule of this size may have difficulty diffusing through the serosa and muscularis to its presumed site of action on the mucosal cells. Also in view of the known lability of purified hormone at neutral pH (8), inactivation of the hormone may occur before it has time to exert an effect. Because of these considerations large doses of hormone were employed, in spite of which inconsistent results have been observed.

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OXYTOCIC ACTIVITY IN THE NEURAL COMPLEX OF TWO ASCIDIANS, *CHELYOSOMA PRODUCTUM* AND *PYURA HAUSTOR*[1]

ABSTRACT

Weak oxytocic activity can be extracted from the neural complexes of two solitary ascidians, *Chelyosoma productum* and *Pyura haustor*. This activity does not resemble neurohypophysial oxytocin pharmacologically or chemically. It can also be found in tissues other than the neural complex. *Pyura* extracts exert a weak antidiuretic effect but *Chelyosoma* extracts do not. The similarity of ascidian neural complex activities to those of the vertebrate neurohypophysis are considered too superficial to support the argument for homology of these organs.

Neurohypophysial extracts characteristically contain oxytocic, vasopressor and antidiuretic activities. This is true of all vertebrate classes including the most primitive, the cylostomes (1, 2, 3). It is tempting, therefore, to look for a structure in invertebrates that might be considered homologous to the neurohypophysis. Butcher (4) reported that an extract of the neural complex of *Molgula manhattensis* contains oxytocic activity equivalent to approximately 55 milliunits per complex. Bacq and Florkin (5) found pressor activity corresponding to about 8 mU./complex in *Ciona intestinalis* and oxytocic activity estimated to be something less than 7 mU./complex. These observations have been accepted as supporting the argument that the ascidian neural complex is homologous with the vertebrate neurohypophysis (6).

Pérès (7) confirmed the existence of oxytocic activity in the neural complex of *Ciona* but found it in other tissues as well. He believed that this activity resembles that of histamine and its ubiquity argues against homology between neural complex and neurohypophysis. His findings on *Ciona* have been confirmed recently by the Dodds (8) who also found that the oxytocic material differed significantly from oxytocin both chemically and pharmacologically.

The report of neurosecretory material in the cells of the cerebral ganglion of ascidians by Dawson and Hisaw (9) has again raised the question of possible homology with the vertebrate neurohypophysis. The present study was undertaken to examine some of the pharmacological properties of extracts of neural complexes from two large solitary ascidians that can be obtained in adequate numbers off the coast of northwestern Washington.

METHODS

Chelyosoma productum and *Pyura haustor* were dragged from the bottom of the waters surrounding the San Juan Islands, mostly from the mouth of the West Sound of Orcas Island, Washington. They were kept in floating live cars and dissected as soon as possible. Over 2400 neural complexes were isolated. Each was dropped immediately into acetone. After 50 complexes were collected the acetone was changed and the material placed in the refrigerator for at least 24 hours. The acetone was then poured off and the complexes were dried in a vacuum desiccator over phosphorus pentoxide in the cold. Dried complexes were homogenized in 0.25% acetic acid and extracted by heating in a boiling water bath for five minutes. Other tissues were treated similarly. All extracts were neutralized to pH 7.0–7.6 before assay.

Oxytocic assays were performed on isolated uteri from rats pretreated with estradiol benzoate, 10 μg. per day for at least three days. Spontaneous motility was suppressed by using a low (32° C) bath temperature and low calcium Locke's solution (10).

Antidiuretic activity was estimated by intravenous injection into ethanol anesthetized rats maintained at a constant level of hydration after the technique of Dicker (11). Pressor assays were performed as described in U.S.P. XV on chlorbutanol anesthetized rats pretreated with Dibenamine (N, N-dibenzyl-β-chlorethylamine hydrochloride, Smith, Klein and French) intravenously (12). The standard used for the oxytocic assay was Pitocin (Parke, Davis and Co.). Pitressin (Parke, Davis and Co.) was used for the antidiuretic and pressor assay standards. The Pitocin was standardized against an U.S.P. Standard Posterior Pitui-

[1] This work was made possible by a Lalor Foundation Faculty Summer Award and was carried out during the tenure of a Lederle Medical Faculty Award.

TABLE 1. ACTIVITIES RESEMBLING NEUROHYPOPHYSIAL HORMONES IN THE NEURAL COMPLEXES OF *Chelyosoma productum* AND *Pyura haustor*. EACH FIGURE REPRESENTS A SEPARATE ASSAY

Species	Equivalent Activity					
	Oxytocic		Antidiuretic		Vasopressor	
	mu./mg.	mu./gland	mu./mg.	mu./gland	mu./mg.	mu./gland
Chelyosoma	0.9 0.8	0.15 0.2	<0.003	<0.003	<0.25	<0.04
Pyura	0.2 0.4	0.1 0.1	0.003 0.007 0.007	0.002 0.002 0.004	<0.5	<0.25

tary Powder by rat uterus assay. The Pitressin was assayed similarly by rat vasopressor and i.v. rat antidiuretic methods. Units shown in the tables are corrected to correspond to equivalent units of the U.S.P. Standard.

RESULTS

Neural complexes from both *Chelyosoma* and *Pyura* contain weak oxytocic activity (Table 1). This activity does not resemble that of oxytocin in the following respects:

1) The response to neural complex extract does not follow a regression parallel to that of oxytocin when plotted against the logarithm of the dose.
2) The sensitivity to neural complex extract varies independently from the sensitivity to oxytocin either with time on a single uterus or between different uteri.
3) The oxytocic activity of the neural complex extract is not appreciably diminished by exposure to 1N NaOH for three hours at room temperature. Such treatment inactivates oxytocin.
4) The oxytocic activity of the neural complex is not reduced by incubation for three hours in 0.01N sodium thioglycollate. This treatment inactivated oxytocin when tested simultaneously.

The activity of the neural complex extracts is almost certainly not due to the presence of oxytocin. The expression of activity in equivalent oxytocic units in the tables is intended only to afford a basis for comparison and is justified only by this necessity.

Oxytocic activity is not confined to the neural complex but can be found in similar concentrations in extracts of other tissues of these ascidians (Table 2). This observation has also been made on *Ciona* by Pérès (7) and the Dodds (8). This is further evidence against the specificity of this activity and argues against any direct relationship to neurohypophysial oxytocin.

TABLE 2. DISTRIBUTION OF OXYTOCIC ACTIVITY IN THE TISSUES OF *Chelyosoma productum* AND *Pyura haustor*

Tissue	Equivalent activity mu./mg. dry weight	
	Chelyosoma	*Pyura*
Neural complex	0.8, 0.9	0.2, 0.4
Cerebral ganglion	1.0, 0.8	
Subneural gland	1.2, 3.0	
Basket	present	0.2
Tunic	present	
Mantle		0.4
Gonad	0.2	0.1
Muscle	1.0	
Blood	<0.04	

Not only does the oxytocic activity of ascidian tissue fail to resemble neurohypophysial oxytocin but it does not resemble other known oxytocic agents that might be present in tissue extracts. It cannot be histamine because histamine depresses rather than stimulates the contraction of the rat uterus. Substance P seems unlikely since it is destroyed by exposure to 1N NaOH (13) whereas the ascidian extract activity is unaffected by prolonged exposure to alkali. It cannot be acetylcholine since it is not blocked by concentrations of atropine that completely inhibit the response of the uterus to acetylcholine. Serotonin (5-hydroxytryptamine) is ruled out since the response to ascidian extract is not blocked by concentrations of atropine or lysergic acid diethylamide[2] that effectively inhibit the oxytocic action of serotonin.

There is no detectable antidiuretic or vasopressor activity in extracts of *Chelyosoma* complexes (Table 1). *Pyura* complex extracts produced no pressor response but did evoke a reproducible antidiuretic response. This response is so weak, however, when one considers the relatively large quantity of foreign tissue extracts injected, it seems unlikely that it represents any degree of specificity. This level of antidiuretic activity is less than 1/200,000 that of the standard dried mammalian posterior pituitary powder.

DISCUSSION

The observation that many tissues of *Chelyosoma* and *Pyura* contain weak unidentified oxytocic activity is in agreement with the findings of Pérès (7) and the Dodds (8) on *Ciona*. This oxytocic activity is certainly not closely related to neurohypophysial oxytocin. The oxytocic and vasopressor activities in neural complex extracts reported by Butcher (4) and Bacq and Florkin (5) may merely reflect the presence of unknown materials in ascidian tissues that can mimic some of the pharmacological properties of neurohypophysial extracts. The resemblance of the ascidian neural complex activities to those of the vertebrate neurohypophysis appears to be too superficial to give support to the argument for homology of these organs.

Acknowledgments

I wish to express deep appreciation for the advice and encouragement provided by Dr. F. L. Hisaw and Dr. F. L. Hisaw, Jr. during this investigation and for the generous contribution of many hours of painstaking dissection of neural complexes by these two and by Mr. David Crockett.

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SEASONAL VARIATION IN THE INCIDENCE OF SPONTANEOUS OVULATION IN RABBITS FOLLOWING ESTROGEN TREATMENT[1]

ABSTRACT

During the winter and spring a considerable number of mature estrogen-treated female rabbits ovulated in the absence of coital stimulation. Such spontaneous ovulation following estrogen administration was not observed during the summer and early autumn months.

The rabbit seldom ovulates in the absence of coital stimulation (1), although a few instances of "spontaneous" ovulation have been reported by Walton and Hammond (2) and Pincus (3). Early attempts to induce spontaneous ovulation in the rabbit with estrogen failed (4, 5), although the steroid was shown to lower the threshold for ovulation in response to copper salts (5) and to copulation during pseudopregnancy (6). Spontaneous ovulation was observed in a small series of rabbits treated with both estrogen and progesterone (7).

METHODS AND RESULTS

As controls in experiments on estrogen facilitation of reflex induction of ovulation (8), mature isolated female rabbits were treated on four successive days with 0.08 mg. estradiol benzoate subcutaneously in oil. At laparotomy 48 hours after the last injection some of the ovaries were observed to contain newly ruptured follicles. This "control" series was then extended to encompass all seasons of the year, and it is the basis of the present communication. The results are summarized in Figure 1. The optimal seasons for spontaneous ovulation following estrogen treatment were winter and spring: during March and April, 9 out of 49 animals ovulated, whereas during the summer and fall months none of 48 ovulated (0/26, June; 0/10, August; 0/12, October).

DISCUSSION

Other parallel seasonal variations in rabbit reproductive phenomena have been observed by numerous investigators. Friedman (9) reported that a higher percentage of females would mate in the spring than in any other season. Zondek and Sklow (9) found that electrical stimulation was effective in evoking ovulation in estrous rabbits from January to June but not later in the year. Borasky and Bradbury (11) discovered that plant extracts, which presumably functioned as neurotoxic agents, would ovulate estrous rabbits during winter and spring but not in summer or autumn. Friedman and

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Friedman (12) described a seasonal variation in the rabbit pituitary gonadotropin content, with a high in April. However, they used the estrous rabbit as their assay animal, and Bradbury (13) later reported a seasonal variation in sensitivity of estrous rabbits to pituitary gonadotropin, with spring as the season of maximal sensitivity. Assaying the hypophyses of cottontail rabbits on the rat, Elder and Finerty (14) found little seasonal variation in the gonadotropin content of the female pituitary. It may well be

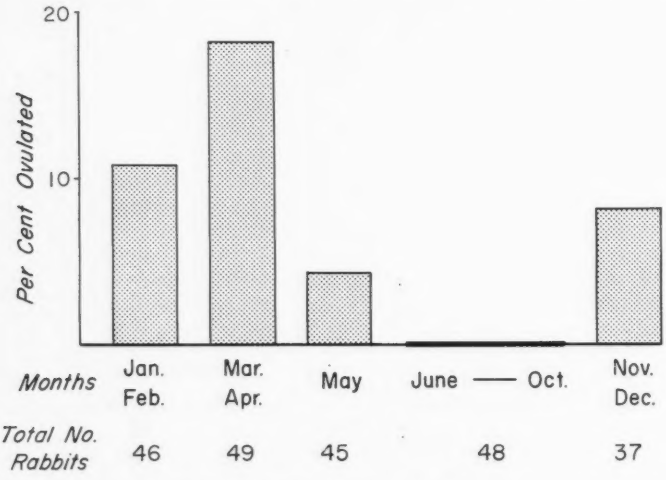

FIG. 1. Relative incidence of estrogen-facilitated spontaneous ovulation in the rabbit during various seasons of the year.

that the important seasonal fluctuation relative to pituitary-ovarian function is not so much a variation in pituitary gonadotropin content as in secretion rate and sensitivity of the pituitary hormone release mechanism.

It seems likely that estrogen, in the rabbit as in the spontaneously ovulating rat, exerts a primary effect on the mechanism for the release of pituitary ovulating hormone. Even in the rat it has been shown (15) that the nervous system participates in the mechanism by which estrogen facilitates release of ovulating hormone, and there is equal reason to expect that the nervous system is involved in spontaneous ovulation in the rabbit. The capacity of estrogen to lower thresholds for the reflex induction of ovulation suggests that at minimal threshold certain afferent nervous impulses not ordinarily effective in inducing ovulation may start the mechanism for pituitary gonadotropin release. The sites and mechanisms of these threshold changes are under investigation by neurophysiological methods (16).

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THE STIMULATION OF LEUCINE-2-C^{14} INCORPORATION INTO THE PROTEIN OF ISOLATED RAT DIAPHRAGM BY SIMIAN GROWTH HORMONE ADDED *IN VITRO*[1]

ABSTRACT

Simian growth hormone, when added *in vitro* in low concentrations, consistently and significantly stimulated the incorporation of leucine-2-C^{14} into the protein of isolated diaphragms of hypophysectomized rats. In contrast, equivalent concentrations of bovine and porcine growth hormone preparations only occasionally produced a similar response.

Bovine growth hormone, when administered chronically *in vivo*, stimulates the incorporation of leucine-2-C^{14} into the protein of isolated diaphragms of hypophysectomized rats (1–2). The present study was undertaken to determine if growth hormone, when added *in vitro*, can also stimulate leucine-2-C^{14} incorporation into muscle protein.

MATERIALS AND METHODS

Hypophysectomized female rats of the Charles River strain weighing approximately 60 gm. were employed in this study. The animals were hypophysectomized 2 to 3 weeks before they were used in experiments, and were fed *ad libitum* until sacrificed. They were killed by a blow on the neck, the diaphragm was rapidly excised and placed into freshly oxygenated buffer. The diaphragm was then carefully divided into two hemidiaphragms by cutting through the central ligament and through the insertions to the spine and xiphoid process. Each hemidiaphragm with ribs attached (weighing about 0.75 gm.) was blotted on filter paper and transferred immediately to a 50 ml. Erlenmeyer flask containing 6.0 ml. Krebs phosphate buffer, pH 7.4, and DL-leucine-2-C^{14} (Tracerlab, Inc., S.A. 0.51 mc./mM) at a concentration of 0.1 mM (25,000 C.P.M./ml. medium). One hemidiaphragm of each pair served as the control, while the other was placed in medium to which growth hormone was added at varying concentrations (see below). The two hemidiaphragms were then incubated, with shaking (120 oscillations/min.), for 90 minutes at 37° C. During the first two minutes of incubation, the flasks were flushed with 100% oxygen and then sealed. The incorporation reaction was terminated by rapidly chilling the flasks to 0° C. The tissue was blotted and spread on a block of ice, and the diaphragm muscle was carefully trimmed of ribs and adhering tissue.

The protein of the diaphragm was prepared by methods previously described (2). The

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yield of protein was about 18 mg./100 mg. of wet muscle. No differences in the yield of protein between the control muscles and those exposed to growth hormone *in vitro* were observed. The dried protein was plated and counted with a gas flow, thin end-window counter, and the radioactivity measurements were corrected for background and self-absorption. The results are expressed as counts/minute/mg. of dried protein. Since pairs of hemidiaphragms were used in these experiments, with one serving as the control for the other, the statistical procedure for paired comparisons was employed to assess the significance of the data (3).

The growth hormone preparations used in these experiments were the following:

a. Bovine growth hormone (NIH-BGH-1).[2]
b. Porcine growth hormone (P205 A).[3] This preparation was derived from the oxycell filtrate, which is the starting material for the Raben-Westermeyer preparation, by a new procedure (4). It is electrophoretically homogeneous and is similar in molecular weight to monkey growth hormone. It has the same N-terminal and C-terminal amino acid (phenylalanine) and it resembles, but is not identical to, simian growth hormone in amino acid composition. It is less soluble than monkey growth hormone in the isoelectric region (pH 5.5). Biologically, it is equipotent with purified simian growth hormone and with highly purified bovine growth hormone in the 10 day weight gain test in hypophysectomized rats.
c. Simian growth hormone (M425 B).[3] This hormone was prepared by a slight modification of the procedure described by Wilhelmi (5).

These hormones were dissolved in distilled water, adjusted to pH 8.0 with 0.1N NaOH, immediately before the start of the experiment. The concentration of hormone was adjusted so that, in each instance, 0.15 ml. of hormone solution, when added to the flask already containing 6.0 ml. buffer, would produce the desired final concentration (1–100 μg./ml. medium). An equal volume of distilled water, adjusted to pH 8.0 was added to the control flasks.

RESULTS AND DISCUSSION

Control experiments were performed to ascertain the variability in leucine-2-C^{14} incorporation between paired hemidiaphragms in the absence of added growth hormone. The data obtained are presented in Table 1.

When bovine growth hormone was added to the medium at a concentration of 1 μg./ml., there was no effect on the incorporation of leucine into protein of the diaphragm (Table 1). At higher concentrations of bovine growth hormone (10–100 μg./ml. medium) there was an apparent stimulation of leucine incorporation, but the response obtained was inconsistent and not statistically significant. The addition of glucose to the incubation medium at a concentration of 200 mg.% did not affect this response. Further experiments with bovine growth hormone (not given in Table 1) were performed in which the experimental conditions were modified by using a shorter incubation time (60 minutes) and, in some instances, Stadie-Zapp buffer (6) rather than Krebs phosphate buffer. Under these conditions, 18 hemidiaphragm pairs were incubated with bovine growth hormone at concentrations of 50–100 μg./ml. medium, and again suggestive but statistically insignificant data were obtained.

The isoelectric point of the bovine growth hormone preparation employed in the above experiments is pH 6.9, which is relatively near the pH of the buffer in the incubation medium. In view of the possibility that the solution of this preparation may not have been complete in the medium employed, the experiments were repeated with similar concentrations of a porcine preparation with equivalent growth promoting properties but with a lower isoelectric point. The results of these experiments are presented in Table 1. There was no statistically significant effect on the incorporation of leucine-2-C^{14} into muscle protein when porcine growth hormone was added to the medium in concentrations of 1–100 μg./ml. medium. These findings would suggest that the failure of

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[2] The bovine growth hormone was a gift of the Endocrinology Study Section of the National Institutes of Health.

[3] We are indebted to Dr. A. E. Wilhelmi for generous supplies of the porcine and simian growth hormone preparations.

TABLE 1. EFFECTS OF *in vitro* ADDITION OF BOVINE, PORCINE AND SIMIAN GROWTH HORMONES ON THE INCORPORATION OF LEUCINE-C^{14} INTO THE PROTEIN OF DIAPHRAGMS OF HYPOPHYSECTOMIZED RATS

Growth hormone (μg./ml.)		No. diaph. pairs	C.P.M./mg. protein			P
			Control	Exper.	Diff. ±S.E.	
	0	12	173‡	169	− 3.53± 9.56	0.8
Bovine	1	4	199	193	− 5.93±20.2	0.8
	10	4	155	222	66.8 ±21.2	0.1
	50*	8	209	234	23.5 ±12.1	0.09
	100	4	183	237	54.0 ±21.7	0.1
Porcine	1	4	230	256	25.7 ±21.8	0.4
	10	4	211	238	26.9 ±16.4	0.2
	100	4	216	254	38.6 ±34.9	0.4
Simian	1	4	164	208	44.4 ±31.7	0.3
	10	9	163	254	82.2 ±17.7	0.01
	10†	6	182	230	48.0 ±11.6	0.01
	100	4	189	289	100.1 ±20.9	0.02

* Glucose present in medium at a concentration of 200 mg.%.
† Equimolar bovine serum albumin added to control medium.
‡ Mean value.

the bovine growth hormone preparation to significantly stimulate leucine incorporation *in vitro* was probably not related to its solubility in the medium.

When simian growth hormone, equipotent with the bovine and porcine hormones (see Materials and Methods) was added to the medium in concentrations of 10–100 μg./ml., a consistently significant increase in the incorporation of leucine-2-C^{14} into diaphragm protein was observed. The response obtained does not appear to be a nonspecific protein effect, since the addition of bovine serum albumin to the control medium did not abolish the difference in leucine incorporation between control and hormone treated hemidiaphragms. The response was not evident, however, when the concentration of simian growth hormone was reduced to 1 μg./ml. medium.

From these results it is clear that simian growth hormone, when added *in vitro* in low concentrations, consistently and significantly stimulates the incorporation of labeled leucine into muscle protein. This same response was observed only occasionally with bovine and porcine growth hormones. While the discrepancies between the *in vitro* activities of bovine, porcine and simian growth hormones are of considerable interest, the possibility remains that a contaminant present to greater or lesser degrees in the preparations used may account for the effects observed.

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PROGESTIN IN THE AMNIOTIC FLUID OF MONKEYS[1]

ABSTRACT

Samples of amniotic fluid of 22 monkeys were obtained at Caesarean section on the 40th to 150th day of pregnancy and were bio-assayed for progestin. Specimens from ten monkeys contained 1.70, 1.13, 0.95, 0.57, 0.57, 0.57, 0.57, 0.38, 0.38, and 0.19 Progesterone Equivalents of "free" progestin/ml. amniotic fluid. The remaining 12 samples contained less than 0.19 P.E./ml.

Specimens of amniotic fluid were obtained from 22 monkeys (*Macaca mulatta*) during Caesarean section on the fortieth to one hundred fiftieth day of gestation. The samples were promptly frozen. Later each was thawed and thoroughly stirred, and 1 ml. was removed and added, drop by drop with constant stirring, to several volumes of cold acetone. The mixture was centrifuged. The precipitate was then suspended in a cold mixture (1:1) of acetone and ether, the resulting material was re-centrifuged, and the supernatant was set aside; this procedure was carried out three times. The original and all succeeding supernatants were pooled, a measured volume of sesame oil was added, the acetone and ether were removed by evaporation, and the residue was centrifuged to separate the oil and water phases. The latter was discarded, and the oil was bio-assayed by the method of Hooker and Forbes (1).

"Free" (i.e., ether- and acetone-soluble) progestin present was measured in Progesterone Equivalents (P.E.). One equivalent is defined as equal to the *activity* of 1 μg. progesterone when the latter is assayed by the procedure of Hooker and Forbes in the absence of estrogen and other interfering factors.

Samples from ten monkeys contained 1.70, 1.13, 0.95, 0.57, 0.57, 0.57, 0.57, 0.38, 0.38, and 0.19 P.E./ml. amniotic fluid. The other 12 samples contained less than 0.19 P.E./ml. There appeared to be no correlation between the stage of pregnancy and the amount of "free" progestin detected.

Zander and von Münstermann (2), using a physico-chemical assay, found progesterone in each of ten samples of human amniotic fluid obtained during the second to tenth months of pregnancy. The amount of this hormone ranged from 0.50 to 0.01 μg./ml. amniotic fluid, the average being 0.14 μg./ml. The Hooker-Forbes method is sensitive not only to progesterone but also to Δ^4-3-ketopregnene-20α-ol and Δ^4-3-ketopregnene-20β-ol (3, 4). The somewhat higher values for monkeys may have been due to the presence of the latter compounds, to a synergism between substances in the extract, or to a species difference between monkey and man.

The highest concentrations observed in the amniotic fluid (1.7 μg./ml.) of the monkey are less than the maximum level of progestin (earlier regarded erroneously as being en-

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tirely progesterone) detected by the Hooker-Forbes method in samples of plasma from peripheral vein blood (3 μg./ml.) obtained during pregnancy in this animal (5).

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INCREASED PITUITARY WEIGHT PRODUCED BY STILBESTROL IN INTACT AND CASTRATED RATS

ABSTRACT

Intact and castrated rats were injected with 250 μg. of stilbestrol every other day for periods of 6, 16 and 26 days. It was found that stilbestrol causes statistically significant differences between pituitary weights of the two injected groups given the same dose. It is concluded that the differences in sensitivity between intact and castrated animals depend upon the initial state of pituitary.

The effect of gonadectomy upon the pituitary in rats of either sex is an enlargement of the anterior lobe with corresponding histologic changes. Chronic treatment with large doses of stilbestrol also causes an enlargement of the pituitary in the intact rat (1, 2). No differences could be detected in the increases in pituitary weight produced by large doses of estrone in intact and castrated adult male rats (3). In this study castration was performed 24 or 48 hours before the first injection.

It is of interest, however, that chronic treatment with large doses of stilbestrol acts on preexistent castration changes of longer duration.

METHODS AND MATERIAL

Gonadectomized and intact male and female rats weighing about 120 gm. were left untreated for two months. They were then divided into 4 groups with equal number of males and females castrated and intact rats in each group.

Group I consisted of 16 untreated animals. The remaining rats were divided in 3 groups each consisting of 8 animals (4 castrated and 4 intact) which were injected subcutaneously with stilbestrol in doses of 250 μg. in 0.05 ml. olive oil per rat every other day for periods of 6, 16 and 26 days. Control animals received equal volumes of the vehicle. All the animals were fed *ad libitum*. At the end of each period of treatment the animals were killed by bleeding under ether anaesthesia. The body weight of the animals was recorded immediately after death. The hypophyses were removed, and their weights expressed as proportions of body weight (Table 1).

RESULTS

As can be seen from Table 1, stilbestrol markedly increases pituitary weight in intact rats. In castrated rats, where the initial weight of pituitary is high, there is also an increase but this is much smaller.

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TABLE 1

Groups	Number of animals	Days of observation	Pituitary weights (mg./gm.)	
			Intact rats	Castrated rats
I. Untreated rats	16	—	0.027 ± 0.004	0.045 ± 0.001*
II. Stilbestrol	8	6	0.062 ± 0.001	0.070 ± 0.003*
III. Stilbestrol	8	16	0.170 ± 0.001	0.140 ± 0.001*
IV. Stilbestrol	8	26	0.182 ± 0.010	0.152 ± 0.007*

* Indicates the significance of the deviations observed in all intact rats in comparison with castrated rats, $P < .05$. (Student's "t" test)

Using doses of stilbestrol of 250 μg. per rat every other day we were able to show differences in sensitivity between intact and castrated rats. There were statistically significant differences in weight between the pituitaries of the two injected groups given the same dose. The control animals which received only olive oil did not show any significant changes in comparison with nontreated animals.

DISCUSSION

That estrogen causes increases in pituitary weight has been a generally accepted fact but the difference between pituitary sensitivity to large doses of estrogen by intact and castrated rats described herein has not previously been noted.

The effect of estrone on pituitary weight has been reported to be increased by castration in male rats (4, 5). Simpson and Williams (3) repeated this experiment and could not confirm the findings of Smith and Smith (4, 5). They detected no difference in the increase in pituitary weight produced by estrone injections in intact and castrated adult male rats.

In the present investigation significant differences in the increase in pituitary weight were found during every stage of treatment.

The method used in the study was different from that used by Simpson and Williams (3). The main difference is that the latter workers performed castration 24 or 48 hr. before the first injection. In this study the castration was performed 60 days before the beginning of treatment. Thus, it is likely that the differences in sensitivity between intact and castrated animals depend upon the initial state of the pituitary. In castrated animals the initial weight of the pituitary is high and sensitivity to stilbestrol is decreased. This explanation receives some support from the fact that when the endogenous source of estrogen is removed by ovariectomy there are practically no mitoses present in the anterior hypophysis (6). According to Payne (7) castration in fowl brings about certain well known changes in the pituitary. Capons 4 to 6 years old show greater changes than cocks 9 years of age. Hunt (8) has shown that the mitotic activity of the anterior hypophysis declines with advancing age, and that the capacity of estrogen to stimulate this mitotic activity also decreases as rats become older (9).

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THE ENDOCRINE SOCIETY

1960 Annual Meeting

The Forty-Second Annual Meeting of The Endocrine Society will be held in the Eden Roc Hotel, Miami Beach, Florida, Thursday, Friday, and Saturday, June 9, 10, 11, 1960.

The Chairman of Local Arrangements is Dr. Kenneth Savard.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 10 at 7:30 P.M. preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Eden Roc will hold 250 bedrooms and the adjoining hotels, the Montmartre Hotel and Lucerne Hotel will hold 75 rooms each, for members until May 1, 1960, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations now directly with the hotel, advising them of time and date of arrival and departure. If you plan on remaining through June 12th for the American Medical Association and other meetings, it is imperative that your reservation be made from June 12th on, directly through the A.M.A. Housing Bureau, c/o Miami Beach Convention Bureau, 1700 Washington Avenue, Miami Beach, Florida, since no reservation beyond this date can be accepted directly by the hotels. Make your reservations now and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1960.

Final program, membership card and advance registration forms will be sent on May 1st 1960 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send an original and four copies of the title and abstract to the Vice-President, Dr. Robert H. Williams, University of Washington School of Medicine, Seattle 5, Washington, not later than February 15, 1960. It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately two hundred abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee. THE COUNCIL REQUESTS THAT AUTHORS ADHERE STRICTLY TO THE FOLLOWING RULES WHEN SUBMITTING ABSTRACTS, OTHERWISE THEY CANNOT BE CONSIDERED:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.

2. Abstracts may not exceed two hundred words, or equivalent space' exclusive of title. No footnotes or acknowledgements to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formula cannot be used.

3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words;
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)". Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)". The principal degree, e.g., M.D., of each author should be written after his name.

   Line 3. Institution of origin and city in which institution is located.

4. Authors who wish receipt of their abstracts acknowledged should enclose self-addressed post card with the title of the abstract noted thereon.

5. Authors are also to enclose 3×5 filing cards, one for each author, giving his name and initials in the upper right hand corner, and the complete title of the abstract with all the authors listed in the center of the card. There should be a second card enclosed listing the title and all authors and mailing address of the senior author. This is necessary for indexing. Senior authors will be notified of the acceptance of their papers for the final program.

6. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. The original and four copies should be forwarded to Dr. Williams.

7. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

1960 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Fred Conrad Koch Award

During the past year a substantial legacy has been bequeathed the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957, Dr. I. L. Chaikoff in 1958 and Dr. Wilbur W. Swingle in 1959. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer, and 1959 Dr. Gordon L. Farrell. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology.

Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and the Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The awards will not exceed $2,500 annually for each individual and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award; the Ciba Award; and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than October 15, 1959.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by October 15, 1959.

The Awards Committee will meet in October and notice of awards to successful nominees and applicants will be made not later than December 1st.

FIRST INTERNATIONAL CONGRESS OF ENDOCRINOLOGY

COPENHAGEN, JULY 18–23, 1960

Details for travel arrangements and general information concerning the program are noted in the June and July issues of ENDOCRINOLOGY.

Registration and opening of the Congress takes place on Monday, July 18th, 1960.

The scientific program consists of ten symposia, a round table discussion and short communications related to the subjects of the symposia.

Symposia:

To the symposia are invited eminent speakers and discussers. At four three-hour symposia there will be four invited speakers and four invited discussers, and at six two-hour symposia there will be three invited speakers and three invited discussers. The symposia comprise:

Tuesday, 9–12 A.M.: Central nervous system regulation of anterior pituitary secretion (ACTH, TSH, gonadotrophins).

Tuesday, 1–3 P.M.: Posterior pituitary hormones (chemistry, biological effects).

Tuesday, 3:30–5:30 P.M.: Parathyroid hormone and calcium metabolism (chemistry, biological effects, clinical aspects).

Wednesday, 9–12 A.M.: Anterior pituitary hormones (chemistry, biological effects, clinical aspects).

Wednesday, 1–3 P.M.: Adrenocortical syndromes (chemical, biological and clinical aspects).

Wednesday, 3:30–5:30 P.M.: Aldosterone (control of secretion, mode of action, clinical aspects).

Thursday: Off-day (for excursions, etc.).

Friday, 9–12 A.M.: Aspects of reproduction (developmental aspects, male hypogonadism, hormones in pregnancy, chemical contraceptives).

Friday, 1–3 P.M.: Steroid pharmacology (chemistry, biology, clinical aspects).

Friday, 3:30–5:30 P.M.: Hormone action at the cellular level (six invited speakers).

Saturday, 9–12 A.M.: Topics in comparative endocrinology (tumors, insect hormones, invertebrates, vertebrates).

Round table discussion (Saturday afternoon):

The subject of the round table discussion is "The Validity of Hormone Assays Applicable to Clinical Medicine" and comprises Aldosterone, Corticosteroids, Progesterone, Androgens, Oestrogens, Gonadotrophins, Thyrotrophin, Thyroid hormones and Catecholamines. Each subject will be covered by one principal speaker and three subsidiary speakers.

Short communications:

Simultaneously with the symposia a number of sessions will be held for

the presentation of short communications (of ten minutes duration) related to the subjects of the symposia.

Registration and Announcement of Communications

In August–September 1959 the Congress Secretariat will send out to all having previously contacted the Secretariat, forms for registration together with forms for the submission of abstracts of short communications. The abstracts must be in our hands before December 31st, 1959. *Members are requested not to submit titles of short communications or advance abstracts before the receipt of these forms.* Anyone interested in participation in the Congress who has not previously submitted his name to the Congress Secretariat is requested to do so at his earliest convenience.

Dr. Svend G. Johnsen, *Congress Secretariat*
Hormone Dept., Statens Seruminstitut
Copenhagen S, Denmark

MEMOIRS OF THE SOCIETY FOR ENDOCRINOLOGY

NO. 6. IMPLANTATION OF OVA

The attention of members of the Endocrine Society is drawn to the publication of this Memoir which reports the proceedings of a Symposium held in London to survey nidation of the mammalian ovum. The various factors—hormonal, biochemical and pharmacological—concerned are discussed in the light of recent research, most of it previously unpublished.

The volume consists of about 100 pages of text and is extensively illustrated with text-figures and plates.

Members may obtain a single copy at $4.00 by sending their check to the Treasurer of the Society for Endocrinology, Dr. E. C. Amoroso, Royal Veterinary College, London, N.W. 1. Additional copies may be obtained at a price of $6.00 through booksellers or from the publishers, Cambridge University Press, 32 East 57th Street, New York 22, N. Y.